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Eighth New Drug Seminar: L-Asparaginase and Daunorubicin

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Begin the article with a short summary.

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Contents

Cancer Treatment Reports
Vol. 65, Supplement 4

ARTICLES

Page

Introduction to New Drug Seminar: Daunorubicin and L-Asparaginase 1
Raymond B. Weiss and Vincent H. Bono
Walter Reed Army Medical Center, Washington, DC

The Discovery of Daunorubicin 3
A. Di Marco, G. Cassinelli, and F. Arcamone
Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

Toxicology of Daunorubicin in Animals and Man 9
R. J. Maral and M. Jouanne
Rhône-Poulenc Research and Development, Vitry-sur-Seine, France

Risk Factors for the Development of Daunorubicin Cardiotoxicity 19
Daniel D. Von Hoff and Maxwell Layard
The University of Texas Health Science Center at San Antonio

Daunorubicin Treatment of Adult Solid Tumors 25
Raymond B. Weiss and Salvador Bruno
Walter Reed Army Medical Center, Washington, DC

Daunorubicin-Prednisone in the Treatment of Acute Non-Lymphocytic Leukemia 29
Bruce A. Peterson, Clara D. Bloomfield, Athanasios Theologides, and B. J. Kennedy
University of Minnesota Health Sciences Center, Minneapolis

Future Clinical Investigations With Anthracycline Antibiotics in Relation to Daunorubicin 35
Franco M. Muggia, Ronald H. Blum, and James C. Wernz
New York University [illegible]ncer Center, New York, New York

A Review of the Preclinical Antitumor Activity and Toxicology of L-Asparaginase Derived from *E. Coli* 39
Lloyd M. Charles, Jr. and Vincent H. Bono, Jr.
National Cancer Institute, Bethesda, Maryland

Pharmacology of L-Asparaginase and the Effects of Host and Enzyme Modification 47
Jack R. Uren
Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

Page

L-Asparaginase: Human Toxicology and Single Agent Activity in Nonleukemic Neoplasms 57
Charles M. Haskell
Wadsworth Cancer Center, VA Wadsworth Medical Center, Los Angeles, California

Enzyme Therapy of Cancer, Future Studies 61
J. S. Holcenberg
The Medical College of Wisconsin and Milwaukee Children's Hospital

Sequential Studies of Daunorubicin and Adriamycin in Acute Nonlymphocytic Leukemia 67
Peter H. Wiernik
Baltimore Cancer Research Program, National Cancer Institute and the University of Maryland Medical School

Anthracyclines in the Treatment of Acute Nonlymphocytic Leukemias 73
Michel Boiron, Claude Jacquillat, Michel Marty, Marise Weil, and Christian Gisselbrecht
Institut de Recherches sur les Leucémies et les Maladies du Sang, Paris, France

Studies With Anthracyclines in Pediatric Acute Nonlymphocytic Leukemia 77
Ronald L. Chard, Jr.
Children's Orthopedic Hospital and Medical Center, Seattle, Washington

Prognostic Factors in Response to Anthracyclines 83
Kenneth B. McCredie, Michael J. Keating, Gerald P. Bodey, Edmund A. Gehan, Terry Smith, and Emil J. Freireich
The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston

Progress in Acute Myelogenous Leukemia 87
Robert Peter Gale
UCLA School of Medicine

Treatment of Acute Lymphoblastic Leukemia in Relapse: Efficacy of a Four-Drug Reinduction Regimen 93
David G. Poplack, Gregory H. Reaman, and Robert Wesley
National Cancer Institute, Bethesda, Maryland

Optimal Current Treatment of Childhood Acute Lymphoblastic Leukemia 97
Joseph V. Simone
St. Jude Children's Research Hospital, Memphis, Tennessee

L-Asparaginase as a Single Agent in Acute Lymphocytic Leukemia: Survey of Studies from Childrens Cancer Study Group 101
Mark E. Nesbit, Inta Ertel, and G. Denman Hammond
Childrens Cancer Study Group

Clinical Pharmacology of Daunorubicin 109
Robert S. Benjamin
The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston

Page

L-Asparaginase: Discovery and Development as a Tumor-Inhibitory Agent 111
John D. Broome
State University of New York, Downstate Medical Center, Brooklyn

Asparaginase-Methotrexate in Combination Chemotherapy: Schedule-Dependent Differential Effects on Normal Versus Neoplastic Cells 115
Robert L. Capizzi
University of North Carolina School of Medicine, Chapel Hill

Asparaginase and Amino Acids in Cancer Therapeutics 123
James F. Holland and Takao Ohnuma, and
Cancer and Leukemia Group B Investigators
Mount Sinai School of Medicine, New York, New York

Eighth New Drug Seminar on L-Asparaginase and Daunorubicin

Division of Cancer Treatment
National Cancer Institute

December 17, 1979
Bethesda, Maryland

Introduction to New Drug Seminar: Daunorubicin and L-Asparaginase

Raymond B. Weiss[1,*] **and Vincent H. Bono**[2]

SUMMARY

This issue will comprehensively cover the development and clinical evaluation of two drugs that play a major role in the treatment of acute leukemias. These papers provide oncologists and hematologists with a single source of information, both preclinical and clinical, about the chemical nature, toxic effects, and use of these two drugs. In addition, some areas for future study are suggested.

[Cancer Treat Rep 65 (Suppl 4):1–2, 1981]

Daunorubicin was isolated from a *Streptomyces* culture in the early 1960s by Di Marco and his associates in Italy. About the same time, Maral and associates in France independently isolated an identical agent. In the 18 years since its discovery, daunorubicin has been shown to be the single most effective drug for the treatment of acute nonlymphocytic leukemia (ANLL). When daunorubicin is used in combination with cytosine arabinoside, complete response rates well in excess of 50% are achieved. With intensive treatment and modern supportive care, the median survival in ANLL has advanced to approximately 15 months, and there is now a notable fraction (10%–20%) of patients who are disease-free in excess of three years. Some of these patients may represent cures. Prior to the 1970s and the widespread use of daunorubicin, very few patients lived this long free of disease.

Daunorubicin's analog, doxorubicin, is also useful in the treatment of ANLL and will produce equivalent response rates. However, it was the consensus of participants in this seminar that daunorubicin is the preferred drug for several reasons. One is that daunorubicin produces less mucositis. Another is that dose reductions for hepatic excretory dysfunction are mandatory for doxorubicin, whereas this is true for daunorubicin only when severe hepatic dysfunction is present. In addition, daunorubicin may be less cardiotoxic in adults than doxorubicin, although this point has not been fully studied. The current Cancer and Leukemia Group B clinical trial in ANLL comparing daunorubicin and doxorubicin should provide firm data to clarify this question.

Daunorubicin has been extensively tested in ANLL but has had little evaluation in solid cancers in either adults or children. Although it might be useful to study daunorubicin further in certain solid tumors, the wide spectrum of antitumor activity and acceptable toxic effects of doxorubicin have hindered interest in doing so.

L-Asparaginase was found by Broome in 1961 to be the active ingredient of guinea pig serum that eight years before had been demonstrated by Kidd to inhibit neoplasm growth in mice. Since this agent has a mechanism of action that exploits a metabolic difference between normal cells and cancer cells, its discovery offered great promise for its use as an antineoplastic drug. This promise was enhanced by its general lack of myelosuppressive effect. The hope was dissipated when L-asparaginase was found to have antitumor effect only in acute lymphocytic leukemia (ALL) and closely related diseases such as T-cell lymphoma and lymphoid-variant blast crisis of chronic myelogenous leukemia. It is inactive in solid tumors.

Although its initial promise was not borne out, L-asparaginase has a meaningful role in ALL treatment. It adds only slightly to the already high complete remission rate produced by the vincristine-prednisone combination, but it prolongs the duration of these complete remissions. A combination of vincristine, prednisone, and L-asparaginase is one of the

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preferred regimens for remission induction of ALL. L-Asparaginase is best administered after vincristine and prednisone therapy is completed.

L-Asparaginase has little myelosuppressive toxicity, but one of its major toxic effects is hypersensitivity reactions. These reactions appear to be reduced in frequency by intramuscular administration of the drug, but one must still be prepared to deal with immediate hypersensitivity reactions whenever the drug is being used. If hypersensitivity does occur, L-asparaginase from *Erwinia caratovora*, which is completely different antigenically, can be substituted for the drug derived from *Escherichia coli*. *Erwinia* L-asparaginase is an investigational agent. It is available for use in individual patients and is supplied by the Investigational Drug Branch of NCI.

As part of this New Drug Seminar, two participants were asked to review the optimal current management of the two major forms of acute leukemia, ANLL and ALL. Both authors emphasize the progress that has been made in treatment results for these diseases and indicate where problem areas exist for future studies.

The Discovery of Daunorubicin

A. Di Marco,[1,*] G. Cassinelli,[2] and F. Arcamone[2]

SUMMARY

The authors summarize the early studies on the isolation, structure determination, biological effects, and mechanism of action of daunorubicin (DNR). In fact, the relationship between affinity for DNA and biological activity was first recorded for DNR and later confirmed on several DNA analogs, both biosynthetic and semisynthetic. A possible divergence was eventually observed with 4-methoxy-DNR. This compound is as active as DNR on the DNA synthesis but eight times more potent on different experimental tumor systems. In this context, it is worth noting that recent data on the *N*-acetyl derivatives of DNR and doxorubicin indicated the persistence of some activity on P388 leukemia in spite of their strongly reduced affinity for DNA and cytotoxic activity when compared with the parent compounds.

[Cancer Treat Rep 65 (Suppl 4):3–8, 1981]

HISTORICAL BACKGROUND AND ISOLATION

The history of antitumor antibiotics began in 1940 with Waksman and Woodruff's (1) discovery of actinomycin A, an antibacterial substance produced by *Actinomyces antibioticus.* The disclosure of the antitumor activity of actinomycin C in animal species in 1952 (2) drew closer attention to substances of microbial origin endowed with antitumor activity.

Taking into account the experience already acquired in researching new biosynthetic antibiotics at Farmitalia Laboratories, in 1957, we began investigating antitumor activity in cultures of newly isolated *Streptomyces* or crude isolates and fractions thereof on Ehrlich carcinoma and Sarcoma 180 (S180) in solid and ascites form in mice.

In 1959, we found some antitumor properties associated with a rhodomycin-like anthracycline complex, isolated from cultural broths of a non-identified *Streptomyces* species. This complex displayed a remarkable inhibition of Ehrlich ascites tumor and also induced a significant reduction in the growth of Ehrlich carcinoma and Sarcoma 180 in solid forms at very low doses (0.05–0.5 mg/kg) but did not afford an increase in survival time (3).

These observations and the activity of cinerubins on some experimental tumors described by Ettlinger et al in 1959 (4) indicated the antitumor potential of the anthracyclines. Soon afterwards crude isolates from cultural broths of another *Streptomyces* species displayed significant growth inhibition of experimental tumors and an increase in life span. The microorganism, isolated from a soil sample collected in Apulia, Italy, was recognized as a new species and named *Streptomyces peucetius* (5). The subsequent fractionation studies, which led to the isolation of the main active component, daunomycin, in pure form (6), are outlined in figure 1. Daunomycin was found mainly in the mycelium and the crude extract in acid aqueous acetone was subjected to solvent partition to take advantage of the basic properties of the antibiotic. The subsequent countercurrent distribution and/or partition chromatography on buffered cellulose columns gave pure daunomycin as the hydrochloride in the form of red needles. An antibiotic, named rubidomycin, with similar chromatographic behavior and biologic activity, was independently isolated from *Streptomyces coeruleorubidus* at Rhône-Poulenc Laboratories (7,8). When the identity of rubidomycin with daunomycin was established, the name daunorubicin (DNR) was chosen to reflect the dual origin (9).

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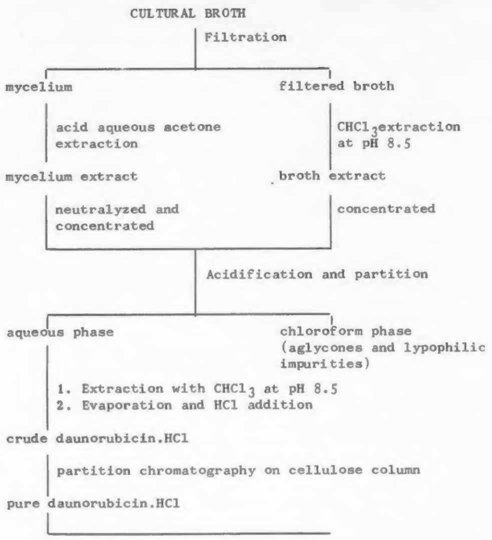
CULTURAL BROTH
Filtration
mycelium
filtered broth
acid aqueous acetone extraction
$CHCl_3$ extraction at pH 8.5
mycelium extract
broth extract
neutralyzed and concentrated
concentrated
Acidification and partition
aqueous phase
chloroform phase (aglycones and lypophilic impurities)
1. Extraction with $CHCl_3$ at pH 8.5
2. Evaporation and HCl addition
crude daunorubicin.HCl
partition chromatography on cellulose column
pure daunorubicin.HCl

FIGURE 1.—Procedure for isolation of daunorubicin from cultural broths.

CHEMICAL STRUCTURE

Our early chemical studies carried out on daunomycin were essentially aimed at ascertaining its chemical features and structural differences with pharmacologically less valuable and already known anthracyclines, mainly studied by Brockmann (10). The name anthracycline is derived from structural features based on an anthraquinone chromophore—related to the tetracyclines—linked to a sugar moiety.

The structure elucidation of daunomycin (I) was based on the chemical studies and the spectroscopic analyses of the aglycone daunomycinone (II) and the amino sugar daunosamine (III) (obtained by mild hydrolysis of I) and their derivatives (fig 2).

In 1964 we assigned a structural formula to the aglycone daunomycinone (II) (11) and the structure and stereochemistry to the amino sugar daunosamine (III) (12). Peculiar features of daunomycin (I), differentiating it from the formerly known anthracyclines, were the acetyl side-chain at C-9, the methoxy at C-4, and the previously unknown amino sugar.

Further studies led in 1967 to the establishment of the location of the substituents and the stereochemistry of daunomycinone, as well as the total absolute configuration of daunomycin (13,14). The result of our efforts not only was the first example of a complete structure determination of an anthracycline, including its absolute stereochemistry by chemical means, but also served as a model for the determination of the absolute stereochemistry of known and new anthracyclines such as β-rhodomycins by Brockmann et al (15), carminomycin by Brazhnikova et al (16), and Adriamycin (doxorubicin) by us (17, 18).

ANTITUMOR ACTIVITY

Di Marco et al (19,20) showed that the crystalline antibiotic (daunomycin) obtained from the cultural broths of *S. peucetius*, if injected by ip route, prolonged the survival time of rodents inoculated with different

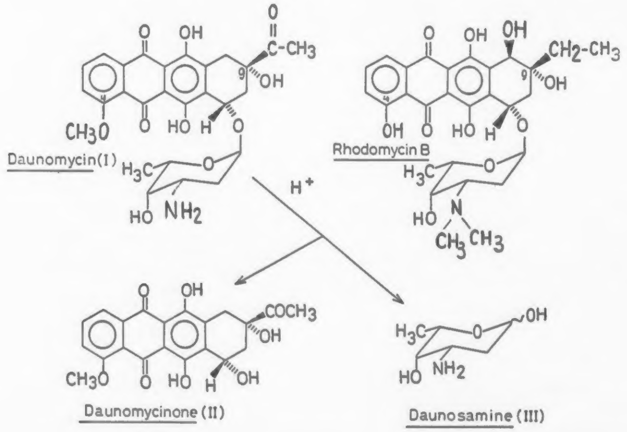
O
OH
O
C-CH3
9
OH
4
CH3O
O
HO
H
O
Daunomycin(I)
H3C
O
HO
NH2
H+
O
OH
OH
CH2-CH3
9
OH
OH
O
HO
H
O
Rhodomycin B
H3C
O
HO
N
CH3
CH3
O
OH
COCH3
OH
CH3O
O
HO
H
OH
Daunomycinone (II)
H3C
O
OH
HO
NH2
Daunosamine (III)

FIGURE 2.—Acid hydrolysis of daunomycin (I) and comparison of its structure with that of rhodomycin B.

tumors in ascitic form (eg, Yoshida 130 hepatoma, Walker 256 carcinoma, S180, Ehrlich ascites carcinoma, and Oberling Guerin-Guerin myeloma). Some solid tumors such as S180, Walker adenocarcinoma, and methylcholanthrene-induced sarcoma were sensitive to the effect of daunomycin (21).

In studies by Laustalot et al (22), daunomycin exerted a selective effect against neoplastic growth in rats that received simultaneous transplants of the Walker 256 carcinoma in solid form, and a benign granuloma caused by cotton pellets. Two different murine leukemia lines, such as a transplantable lymphoma originally induced in C3H mice by the Moloney leukemia virus, and a spontaneous leukemia of CW1 mice, were not sensitive to daunomycin.

At the same time Dubost et al (8) at Rhône-Poulenc published their findings on the activity of rubidomycin on different tumor lines, including some murine leukemias.

Another keystone in the development of DNR was the demonstration of its favorable effects on two systems of great predictive preclinical value: the L1210 and P388 leukemias transplantable in BDF_1 mice (23). In the same work two other important facts emerged: 1) the persistence of the antitumor effect on the L1210 line resistant to methothrexate and cytoxan; 2) the presence of an antitumor effect on S180 solid tumor at a dose that was toxic in normal mice. Other important results of these and subsequent research were the demonstration of a route-dependency of activity and the influence of administration schedule.

Although a number of antitumor agents of microbial origin were known at that time, only for a few (eg, actinomycin D and mitomycin) was there some knowledge of the mode and mechanism of action. A valuable contribution to these studies was the demonstration of a cytotoxic effect of daunomycin exerted at a low dosage (10–100 ng/ml) on different cell lines in tissue culture (24). Owing to the fluorescence of the antibiotic, it was possible to follow the cellular uptake and the subsequent concentration in the nucleus. Cellular damage was predominantly nuclear, at the nucleolar level in the non-dividing cells, with strong mitotic aberrations in cells which reached the mitotic stage (25). The results posed the question as to whether important anomalies in these essential structures were a consequence of effects on the nucleic acid structure and functions.

BIOCHEMICAL EFFECTS

Early experiments (26) showed an effect of DNR on quantitative RNA synthesis in a sensitive bacteria; these results were confirmed by the demonstration of the inhibition of ^{32}P incorporation into RNA of bacteria in which the multiplication was arrested by UV irradiation (27), and by the observation of ^{14}C-adenine into RNA (28,29) and of ^{3}H-uridine into nucleolar and less strongly into extracellular RNA (30,31).

An inhibitory effect was also observed in vitro in the DNA-dependent RNA synthesis catalyzed by *Escherichia coli* RNA polymerase (32,33); this effect was competitively antagonized by DNA. A similar inhibitory effect was observed on the in vitro DNA synthesis (32) and on the incorporation of labeled precursors into DNA of eucaryotic cells (28–30).

From the competitive effect of DNA on in vitro DNA-directed DNA and RNA synthesis, the hypothesis was advanced (34) that the formation of a complex between the antibiotic and DNA could hinder the formation of the DNA-polymerase complex or impair the structural changes of DNA required for the initiation of the polymerization.

MECHANISM OF ACTION

The evidence of physicochemical interaction of DNR with DNA was reported in 1965 by Calendi et al (35) who showed: 1) the reduction in specific absorbancy at 470 nm on addition of DNA, 2) the quenching of the specific fluorescence by DNA, and 3) the disappearance of the wave of polarographic reduction characteristic of anthraquinones. The spectral changes as well as the disappearance of the ability to take part in oxidoreductions indicate the binding of the aromatic hydroxyls of the chromophore to DNA.

On the other hand, there is evidence for an electrostatic linkage of DNR to DNA through the amino group of daunosamine since the capacity of *N*-acetyl-DNR to bind to DNA is decreased.

The double nature of the binding to DNA resulted also from the effect on the complex of compounds, such as formamide, able to split hydrogen bonds: a complete splitting of the linkage between *N*-acetyl-DNR and DNA was observed by spectrophotometry, whereas that between DNR and DNA is only partially broken and the antibiotic is still bound to DNA, as indicated by its sedimentation in ultracentrifuge.

Further evidence of the interaction of DNR with DNA is the protective effect of DNR against thermal denaturation of DNA (35), the depression of buoyant density of DNA of *Sarcina lutea* observed in cesium salt gradients (34,36), the sedimentation with DNA in ultracentrifuge (26,35), and the effect on sedimentation coefficient and specific viscosity of DNA (36).

Planar bases, such as acridines, may bind to DNA with a salt-susceptible bond, related to the hydrophobic interactions of the ring system with adjacent base pairs (37); a similar intercalation binding of DNR to DNA was therefore suggested by Ward et al (33). This was later translated by Pigram et al (38) into a molecular model on the basis of the x-ray diffraction of the fibrous DNR-DNA complex. Neidle (39) provides an up-to-date discussion of this molecular model.

How closely are the biochemical effects on nucleic acid synthesis related to the ability of DNR to form a complex with DNA and to the cytotoxic and antitumor effects? It should be clear at once that we could not expect a simple relationship between the molecular and organismic levels, due to parameters such as cellular uptake, intracellular distribution, tissue distribution, and metabolism. However, if we could find substances unable, or with a significantly reduced ability, to bind to DNA but with significant effect on DNA synthesis and antitumor activity, the theory would be dismissed.

The available DNR analog was *N*-acetyl-DNR, in which the *N*-acetylation considerably reduces the affinity to DNA (35). The inhibitory effect of this compound on DNA synthesis and the cytotoxic activity was strongly reduced, even though in the samples available at that time some antimitotic effect was maintained at a dosage 400 times higher than DNR. In recent studies with pure chemical preparation, a slight activity of this analog on P388 leukemia was observed[3]. Some more recent data on the quantitative relationship between DNR and the *N*-acetyl derivative are reported in table 1. The cellular uptake of the analog is also strongly reduced in comparison with DNR[4].

From the data available in 1965 it was concluded that a reduction in ability to bind to DNA was paralleled by a strong reduction of inhibitory effect on nucleic acid synthesis and antimitotic activity (that could also be ascribed to residual amounts of DNR in the sample). The persistence of an in vivo activity in the *N*-acetyl derivative of DNR, and more consistently of *N*-acetyl-doxorubicin, may likely be ascribed to a metabolic effect of the organism with liberation of the parent antibiotic or of a metabolic active product.

The hypothesis that all DNR and doxorubicin compounds with biological activity have a high affinity for the DNA, and that a reduction of this binding ability leads to a reduction or disappearance of the antitumor effect, has been verified in 100 synthetic analogs of DNR and doxorubicin (table 2) (40).

However, the mechanism by which the binding to DNA is expressed in blockage of nucleic acid synthesis and cytotoxicity is still unclear and some observations

TABLE 1.—Quantitative relationship between DNR and the *N*-acetyl derivative*

Compound	K_{app}	n_{app}	ID$_{50}$ HeLa (ng/ml)	OD P388 ip mg/kg	T/C
DNR	1.3×10^6	0.17	7.2	2.9	154
N-Acetyl-DNR	1.8×10^4	0.12	1600	200	131
Doxorubicin	3×10^6	0.19	6.25	4.4	159
N-Acetyl-doxorubicin	5×10^5	0.03	600	100	172

*Di Marco A, Casazza AM, Zunino F, et al. Unpublished data.

TABLE 2.—Comparison of the biological and biochemical effects of 4′-epi-derivatives of DNR and doxorubicin (ADR) and of their stereoisomers

	Concentration (μm) required for 50% inhibition					Antitumor activity	
Compound	DNA polymerase	RNA polymerase	^{3}H-TdR incorporation*	MEF proliferation†	ΔTm °C	Dose‡	T/C§ %
DNR	16	8	0.37	0.0156	12.0	5	140
4′-Epi-DNR	12	9	0.26	0.0224	12.4	2.5	150
4′-Epi-DNR (β anomer)	>40	>40	3.19	0.1103	4.8	10	125
ADR	11.5	6	2.07	0.0124	14.5	5–10	~400
4′-Epi-ADR	11.5	6	1.27	0.0124	12.5	10	~400
4′-Epi-ADR (β anomer)	>40	28	>3.50	>0.1770	7.6	20‖	195

*In mouse embryo fibroblasts treated from 18–24 hrs after fetal calf serum stimulation.
†Treatment for 72 hrs. MEF = mouse embryo fibroblasts.
‡Optimal dose (mg/kg) in mice bearing P388 leukemia, treated ip on Day 1.
§Median survival time of mice, over median survival time of control mice, x 100.
‖Maximum dose tested.

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[3] Di Marco A et al. Unpublished data.

[4] Supino R. Personal communication.

do not fit simply into the theory. Phase contrast observation by Di Marco et al (25) revealed an antimitotic effect of DNR when administered in a period of the mitotic cycle when the nucleic acid synthesis is no longer present, or appears at a very reduced rate. In synchronized cultures of rat fibroblasts the largest inhibiting effect on mitotic index (MI), number of labeled cells, and extent of ^{3}H-thymidine incorporation was observed when the cells were treated in the central part of the G_1 phase (41). In rat fibroblast cultures a second point of high sensibility to DNR is just prior to the onset of S phase. In HeLa cells the mitotic activity was strongly depressed with dosages of DNR which have only a slight effect on ^{3}H-thymidine incorporation (31).

Recently, further evidence of a possible divergence between the cytotoxic effect and the inhibitory effect on nucleic acid synthesis has been observed, and involves the effect of DNR and the analog 4-demethoxy-DNR on the entry of quiescent cells in mitotic cycle (table 3). These observations suggest that the determinant for cytotoxic activity appears to be an early process which actually precedes the entry of cells in S phase. Many hypotheses could be advanced on the nature of this process, such as binding to some DNA sequences with regulatory functions (44), but such discussion is outside the aim of this paper.

Strictly related to the cytotoxic effect are the toxic effects (such as chromosome damage) observed on normal cells, and the toxicological effect in myelopoietic and other tissues such as kidney, carefully described by Bertalozzi et al[5], as well as the more recent observation of carcinogenic activity (45). We wish to emphasize that it was the peculiarities of the mode and mechanism of action of DNR that impressed Karnofsky et al in clinical studies of microbial products as antitumor agents at the Sloan-Kettering Institute. Their pioneering observations on remissions obtained in acute leukemia (46) and in some other childhood tumors (47) along with those of Bernard et al (9) at Hôpital Saint Louis in Paris represented the starting point of clinical studies with this anthracycline and its derivatives, such as doxorubicin.

We conclude by noting that the development of this agent represents a good example of open international cooperation in the fight against cancer.

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[5] Bertazzoli C. Personal communication.

Table 3.—Comparison of the biological and biochemical effects of 4-demethoxy-DNR and its stereoisomers

	Concentration (μm) required for 50% inhibition					Antitumor activity	
Compound	DNA polymerase	RNA polymerase	^{3}H-TdR incorporation*	MEF proliferation†	△ Tm °C	Dose‡	T/C %
DNR	16	8	0.60	0.0431	12.9	4	190
4-Demethoxy-DNR	16	14	0.48	0.0088	17.0	0.5	180
4-Demethoxy-DNR (βanomer)	42	19	0.60	0.0151	13.4	8	190
4-Demethoxy 7, 9-diepi DNR	51	37	10.00	0.1960	7.3	80	120
4-Demethoxy 7, 9-diepi DNR (βanomer)	>92	>38	10.00	0.5050	5.6	40	120

*In mouse embryo fibroblasts treated from 18–24 hrs. after fetal calf serum stimulation.

†Treatment for 24 hrs. MEF = mouse embryo fibroblasts.

‡See table 2.

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Toxicology of Daunorubicin in Animals and Man

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SUMMARY

The toxic effects of daunorubicin with specific reference to acute, subacute, and long-term studies in animals are reviewed, and its clinical toxicity is considered. We emphasize the importance of hematologic toxicity and infections when treating with daunorubicin. A summary of the work done on detoxifying activities of certain compounds is presented, and the activities that increase the toxic effects of daunorubicin are discussed. Finally, the immunosuppressive, mutagenic, teratogenic, and carcinogenic activities of daunorubicin are mentioned.

[Cancer Treat Rep 65 (Suppl 4):9–18, 1981]

This report reviews the toxic effects of daunorubicin in experimental animals and in man. Daunorubicin has been studied in the laboratory for approximately 18 years, and for about 15 years it has been used clinically, principally in the treatment of acute leukemia. Therefore, we have a large volume of data at our disposal. In particular, we cite general articles or books by Dubost et al (1), Morrison (2), Julou et al (3), Bernard et al (4), and Von Hoff et al (5).

Because the purpose of toxicologic studies is to provide information for the good conduct of treatment in human therapy, the results of preclinical toxicology studies of anticancer agents must have significant predictive value (6), namely, safe doses for phase I clinical trials and predictive toxic effects. On the whole, we have found good correlation between the data of toxicologic studies and the problems encountered clinically. For example, as early as 1962–1963, cardiotoxicity was observed in rabbits in our research center.

EXPERIMENTAL TOXICOLOGICAL STUDIES

Toxicologic studies (acute and subacute toxic effects) have been performed with various animals: mouse, rat, guinea pig, rabbit, dog, hamster, and monkey. In table 1, acute and subacute toxic effects by iv and ip routes are compared. It should be noted that: 1) The results vary somewhat according to the animal. 2) In both rats and mice, daunorubicin is clearly more toxic by ip route than by iv route. 3) By iv route, daunorubicin is less toxic than doxorubicin, whereas by ip route the reverse is true. The greater toxic effects of doxorubicin have been reported by Ghione (7). 4) The histological lesions provoked by daunorubicin in the hamster have been studied by Farmar (8). 5) Tolerance of daunorubicin has been studied in the rhesus monkey by Scholler and Ball (9) where the cumulative dose of 19 mg/kg caused cardiac, hepatic, renal, and pulmonary lesions. In the cat, Henness et al (10) have observed a retardation of hair growth and alopecia. Acute toxic doses of daunorubicin by buccal administration are very high (300 mg/kg po in the mouse or the rat and 40 mg/kg po in the dog). By the pleural route in the rat, the dose of 0.2 mg/kg is well-tolerated (Ganter, personal communication). Toxic effects depend upon the age and sex of the test animal (11).

Tables 2 and 3 show the results obtained in the rabbit and the dog after 90 doses. Daunorubicin is more toxic in the dog than in the rabbit, but the maximum tolerated dose (MTD) is similar in both (0.25 mg/kg iv). The following should be noted: 1) the presence of serious blood toxicity with atrophy of most hemopoietic organs and thrombocytopenia, 2) renal, hepatic, and gastrointestinal disturbances, 3) marked testicular toxicity with atrophy of spermatogenic tissues, and 4) the irreversible cardiotoxicity particularly in the rabbit.

By lumbar injection at doses of 0.1 mg/kg in the dog and 0.5 mg/kg in the rabbit, daunorubicin causes paraplegia and quadriplegia accompanied by a high rate of mortality (J. Pasquet, personal communication). The most striking effect of daunorubicin noted in toxicity studies in animals is its blood toxicity. In the mouse

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Table 1.—Toxicity (LD50 mg/kg)*

Toxicity	Number of treatments	Species	Route of treatment		Remarks
			iv	ip	
Acute	1	Mouse	15–20	3.5–5.7	With ip dosage: hemorrhagic ascite, local cytotoxic effects, clumping of abdominal organs, delayed deaths
			9–15	8–10	Doxorubicin
		Rat	12.5–25	App. 8	
		Guinea pig	App. 6		
		Rabbit	App. 5		
		Dog	4–5		
		Hamster	App. 10		
Subacute	5	Mouse	9–10	1.2–2	
	3 Rest: 3 days Repeat 3 courses	Monkey	App. 2		MTD: 0.5 mg/kg iv LD50: 24 mg/m² iv Toxic cumulative dose: 14-19 mg/kg iv At 2 mg/kg iv: hemorrhagic lesions in the GI tract. Emesis is often seen

*Morrison RJ. Toxicology report to NCI, Jan 1967.

Table 2.—Toxicity in the rabbit
Route of treatments: iv. No. of daily treatments: 90 [Julou L et al 3]

Mg/kg iv	Lethality	Bone marrow blood	Weight	Liver	Kidneys	Heart	Testes	GI tract	Local vein tolerance
2	Deaths 100% (4-10 days)	Aplasia, pancytopenia, bleeding	Loss			Hemorrhage		Hemorrhage	
1	Some postponed deaths	Leukopenia, anemia (slight)	Loss	Inflam. steatosis	Epithelial toxicity (reversible)	Myofiber toxicity (nonreversible)	Toxicity (reversible)	Toxicity	Edema, necrosis
0.5 − 0.25 (DMT = 0.25)	No death		No loss	Mild steatosis (reversible)	Mild toxicity (reversible)	Mild toxicity	Mild toxicity (reversible)		Edema

(fig 1), for example, a single administration of 10 mg/kg iv causes a rapid drop in the number of white blood cells of the bone marrow, and the doses that inhibit by 50% the incorporation of tritiated thymidine into the leucocytes of the bone marrow are: daunorubicin, 4 mg/kg iv, and doxorubicin, 8.5 mg/kg iv. Marsh (12) has studied the effects of daunorubicin and other agents on normal hematopoietic precursor cells (effects on CFU).

Attention should also be drawn to more specific toxicity studies: 1) gastrointestinal toxicity in the rat (13) and in the mouse (14) where the toxicity of carminomycin (prop. INN: carubicin) is greater than that of daunorubicin; 2) hepatotoxicity in the mouse with histopathological damage (15); 3) renal toxicity (16), eg, production of a nephrotic syndrome in the rat after a single dose of 20 mg/kg iv with proteinuria, hyperlipemia, and glomerular damage ("daunomycin nephrosis"); Walski (17) studied the ultrastructural changes in kidney glomeruli in daunomycin nephrosis; and 4) involvement of the endocrine glands. Mazzanti et al (18) have recorded the selective localization of daunorubicin in the pancreas of the rat and the guinea pig. This observation, which has been confirmed by Anderson et al (19), may explain the disturbances of glycoregulation with possible diabetic symptoms during treatment. Davies et al (20) have studied the effect of circadian rhythm on tolerance in the mouse. The optimal treatment time seems to be between 8 AM and noon; the optimum treatment time for daunorubicin differs from that for doxorubicin.

CLINICAL TOXICOLOGY

Bone marrow insufficiency, often encountered, and a group of miscellaneous events, which are of less impor-

TABLE 3.—Toxicity in the dog

Route of treatment: iv. No. of daily treatments: 90 [Julou et al 3, Morrison 2]

Mg/kg iv	Lethality	Bone marrow blood	Weight	Liver	Kidneys	Heart	Testes	GI tract	Local vein tolerance
1	Deaths 100 % (12-13 d)	Leukopenia, granulocytopenia, thrombocytopenia, anemia, bleeding (mouth)	Loss, adynamia, anorexia					Necrotic lesions, hemorrhagic lesions	
0.5	Rare deaths	Leukopenia (mild), granulocytopenia (mild), thrombocytopenia, mild anemia, blood clotting time lengthening (reversible)	Loss, anorexia	Vacuolization of hepatocytes, steatosis, disturbances: urobiline in urine, elevation of SGOT and SGPT	Alteration of tubular epithelium, disturbances: albumin in urine, elevation of urea nitrogen and creatinine		Aplasia	Necrotic lesions in the mouth	
0.25 (DMT = 0.25)	No death						Mild aplasia		

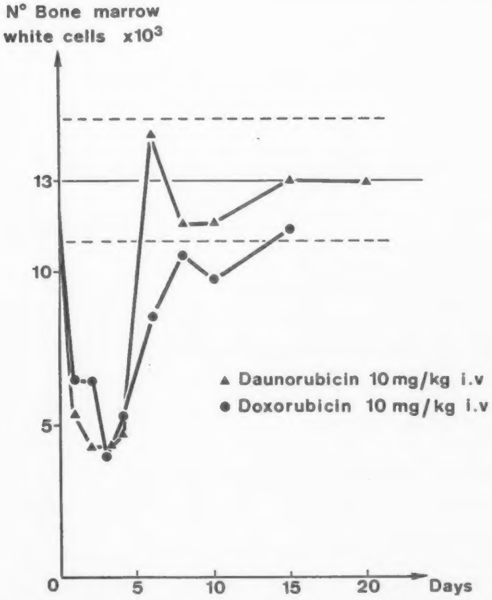

FIGURE 1.—Toxicity for bone marrow white cells in the mouse.

tance because blood and cardiotoxicity are life-threatening, will be considered.

Bone Marrow Insufficiency

Bone marrow and lymphoid depression is seen, especially in the treatment of leukemia. This risk must be accepted, since remission only occurs after the marrow depression phase. During or after treatments (0.75–2.00 mg/kg iv, four to six times per week; cumulative dose 20–25 mg/kg iv), we have an alarming hematological profile that is dose-limiting: 1) cytopenia of the blood, with leukopenia (nadir on Days 8–10 after 3 days' administration, with recovery occurring in an average of 2–3 weeks, or longer in elderly patients), 2) extreme thrombocytopenia with hemorrhagic diathesis and bleeding (21) (nadir 4–15 days after starting therapy with recovery in a few weeks), 3) agranulocytopenia followed by infectious episodes and septicemia (most often caused by gram-negative organisms), and 4) anemia present but not a serious problem.

Transfusion of platelet-rich plasma or platelet concentrates almost always prevents hemorrhage. Bone marrow transplantations are also used for the treatment of blood aplasia (22,23). This presents the problem of graft-versus-host reaction that is a complex undertaking requiring a skilled team of investigators.

Miscellaneous Events

Gastrointestinal Toxicity

The problems encountered are a result of the toxic effects of daunorubicin on the germinal cells of the gastrointestinal tract epithelium. We observe nausea and vomiting that are dose-related. Anorexia and diarrhea (sometimes bloody) are noted. Mucous membrane involvement, with stomatitis and buccal ulcerations, is frequent, but it disappears when the drug is stopped. The bowel is considered the most likely source of endogenous infections.

Fever

Fever may be of different origins. It may be related to disease (the recurrent fever of lymphoma, for example) or to infections. Drug-induced fever usually returns to the baseline after treatment is discontinued.

Vascular Complications

If the infection is not strictly endogenous, after extravasation of daunorubicin we observe local complications due to vein and tissue damage, sometimes very serious with edema, phlebitis, and tissue necrosis. Necrosis of the hand after daunorubicin infusion distal to an arteriovenous fistula has been reported (24). We also note hemostatic complications with disseminated intravascular coagulation after sudden cell lysis caused by treatment with daunorubicin (25).

Allergic Reactions

Contact dermatitis associated with daunorubicin (or doxorubicin) has been observed (26). Allergic reactions are typified by rashes, urticaria, and angioneurotic edema of the lips and the eyes (27). The mechanism of the allergic reactions has been studied in beagle dogs, and the histamine release has been prevented by antihistaminic drugs (28).

Alopecia

Alopecia is related to toxicity for hair follicles. It ranges from thinning of the hair to alopecia; recovery occurs within 2 months.

Hepatitis and Renal Function Disturbances

The manifestations of liver and renal toxicity are rare, but they need to be mentioned because daunorubicin and its metabolites are excreted mainly via the hepatic-biliary route. Liver-function defects and hepatitis are related to an increase of daunorubicin toxicity (29,30). When there is renal damage (tubular nephritis), we observe abnormal function test results: elevation of blood urea nitrogen, hematuria, and the presence of casts.

Hyperuricemia

Hyperuricemia follows sudden cell lysis during chemotherapy for leukemia. It is treated by the use of allopurinol or urate-oxidase and by an increase of diuresis (31).

Infections

Infections present serious complications during treatment with daunorubicin and other drugs; they follow from a state of immunodepression with granulocytopenia and reduced resistance to infection. Infectious agents include: bacteria (often gram-negative), fungi (Candida), parasites, and viruses (herpes zoster). The management of infectious complications is difficult; antibiotics and nonviable vaccines are used. It is preferable, however, to take prophylactic precautions: measures of general hygiene and the use of protected environments such as sterile rooms.

Miscellaneous Toxicologic Activities

We cite only some rare complications: red urine, nail pigmentation (32), and headache. We will not discuss the toxicity of daunorubicin in association with other drugs or the influence of radiotherapy on daunorubicin toxicity. Clinically, the mangement of treatment with daunorubicin poses some problems that must be solved by very experienced teams (33,34). Table 4 gives an overview of the toxic problems encountered clinically when using daunorubicin.

TABLE 4.—Main toxicologic activities of daunorubicin (cardiotoxicity excluded)

	% incidence*
Toxicity for the hemopoietic system	
Leukopenia (< 1500 mm^3)	65
Thrombocytopenia (< 50,000 mm)	54
Anemia	4.6
Gastrointestinal tract toxicity	
Nausea	27
Vomiting	37
Diarrhea	25
Mucositis, stomatitis	15
Fever	33
Phlebitis	30
Skin rash	6
Hair loss, alopecia	35

*Figures from a study of 1156 patients by Von Hoff et al in Advances in Pharmacology and Chemotherapy, New York, San Francisco, London, Academic Press, 1978, vol 15, p 1.

MODIFICATIONS OF DAUNORUBICIN TOXICITY

Detoxifying Activity

It is normal to search for a means of reducing the toxic effects of daunorubicin therapy that retain, or possibly improve, its antitumor activity. The use of diketopiperazines such as the derivative ICRF 159 or razoxane [1,2-*bis*-(3,5-dioxopiperazine-l YL) propane] should be mentioned. This compound, which itself has an antitumor activity (35,36), exhibits a protective activity with regard to the toxicity of daunorubicin, and the association of these two compounds improves the antitumor activity (37–41).

Carriers are also used to detoxify the drug. The ideal carrier is nontoxic, biodegradable, and has affinity for the target site where it releases the active agent. The use of DNA-daunorubicin complex was proposed some years ago by Trouet et al (42). We have found that this complex at low doses is more active than the free drug and at very high doses is less toxic (fig 2). The pharmacology and therapeutic efficacy of the complex in mice were also studied by Ohnuma et al (43). The cumulative cardiotoxicity is lowered or inhibited when the complex is used, and a lower toxicity is also observed for bone marrow, the liver, and the kidney (44). These results were confirmed by Seeber et al (45). Other carriers used with success are liposomes (46,47) or dextran (48). The latter detoxifies and increases antitumor activity.

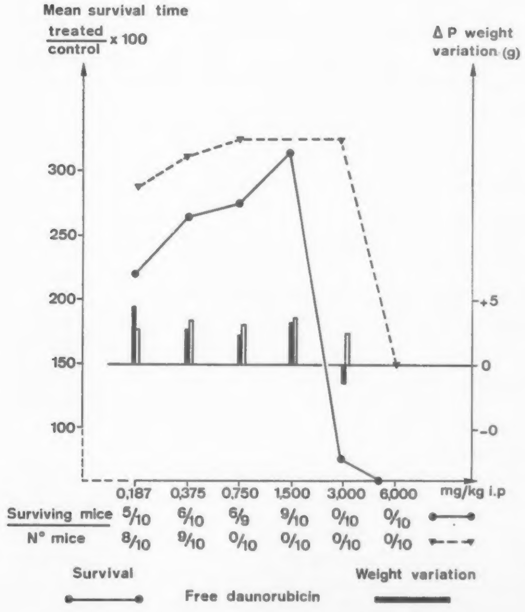

FIGURE 2.—Toxicity of DNA-daunorubicin in mice.

Pretreatment with oxytetracycline has markedly reduced acute toxicity and changed the pharmacokinetic distribution of daunorubicin in daunorubicin-treated mice (49).

In explaining the cytotoxic activity of daunorubicin (or of doxorubicin), recent work accords an important place to the production of free radicals (50,51). During experimental treatment of leukemia P388 with daunorubicin, we studied the possible influence of a bovine superoxide dismutase (SOD). The toxic effects of the anthracycline were decreased and so was its antitumor activity (unpublished data). Kishi, Watanabe, and Folkers (52) have proposed the addition of coenzyme Q 10 as a protector against the toxic effects, particularly the cardiotoxicity, of daunorubicin.

Action Leading to Increased Toxicity

Mitogens

Pokeweed mitogen (PWM) increases the toxic effect of daunorubicin, but PHA does not (53).

Endotoxins

Salmonella typhimurium endotoxin clearly increases the febrile reactions with daunorubicin (54). This must be taken into consideration in cases of treatment with antibiotics of complications arising from infections by gram-negative bacteria. We mention only radiotherapy here.

IMMUNODEPRESSIVE ACTIVITY

The immunodepressive activity of anticancer compounds is an integral part of their toxicology. This activity plays an important role in the tumor-host relationship and is also involved in infectious complications. Numerous studies have been devoted to this topic: decrease of antibody production (4), lowering of delayed hypersensitivity reaction (55), improvement of rat adjuvant arthritis (56), inhibition of interferon production (57), increase of skin-graft survival (58), and inhibition of hemolysis in gel plaque assay (59).

On a tumor that is highly antigenic, the activity of doxorubicin was found to be better than that of daunorubicin. In contrast, on a poorly antigenic tumor, the two drugs were almost without activity (60). According to Schwartz and Grindey (61), after treatment at equivalent doses, daunorubicin was found in the spleen at concentrations about twice those of doxorubicin. It follows that daunorubicin may be more immunodepressive than doxorubicin. According to Schwartz (62), in P388 leukemia in mice, doxorubicin is more active than daunorubicin, but this therapeutic advantage is lost when the host is pretreated with whole-body irradiation. This suggests that daunorubicin is more depressive than doxorubicin. But on C′ dependent and C′ independent in vitro cellular toxicity, no significant difference was found in the effects of the two drugs (63). We have studied the activities of daunorubicin using different immunologic techniques.

Action on Spleen Cells in the Mouse

Plaque assay (hemolysis in gel) is shown in table 5. The number of plaques is reduced after treatment iv with daunorubicin, and, at half-dose iv, doxorubicin is less active. When mice are treated with daunorubicin several days before the culture in vitro of spleen lymphocytes, the response of the lymphocytes to mitogen (PHA, ConA, LPS) is lowered or heightened according to the time schedule of treatment.

TABLE 5.—Spleen cell hemolysis in gel (plaque assay)*

Compound	Mg/kg iv	No. plaques/ spleen assay	No. control / No. treated assay
Control	0	38,691	
Daunorubicin	2.50	8,760	4.4
Doxorubicin	1.25	11,194	3.4

RP XXI mice, females, 6 wks old.
10^9 erythrocytes ip: Day 0.
Treatments, iv route, Days 0, 1, and 2.
Assay: Day 3.

*Jerne NK, Nordin AA, and Henry C. The agar plaque technique for recognizing antibody producing cells. *In* Cell-bound antibodies, Wistar Institute Press, Philadelphia, p 109.

Action on Peritoneal Macrophages in Mice

Using the carbon clearance test (64) we did not find a clear inhibiting activity for either daunorubicin or doxorubicin. In vitro, the concentrations inhibiting 50% of phagocytosis of India ink by peritoneal macrophages in culture are: daunorubicin, 0.12 μg/ml and doxorubicin, 0.34 μg/ml. But, in vivo, when mice are treated with daunorubicin some 3 or 6 days before peritoneal macrophage harvesting and culture with India ink in the medium, the phagocytic activity of the macrophages is heightened. There may be a relation between this stimulus and the increased primary cell-mediated immunity in culture observed by Orsini et al (65). The cytotoxicity of daunorubicin for mouse peritoneal macrophages in culture is higher than that of doxorubicin. (The latter is more toxic than detorubicin.) The tests used were macrophage counting per plate and dosage of cell protein per plate. The concentrations inhibiting 50% of the incorporation of tritiated leucine by macrophages are: daunorubicin, 0.10 μg/ml; doxorubicin, 0.39 μg/ml; and detorubicin, 45 μg/ml. In the graft-versus-host reaction for both daunorubicin and doxorubicin (table 6), there is an increase in the reaction when the route of treatment is intravenous and a decrease when the route is intraperitoneal. The depressive activity of daunorubicin on antitumor activity in mice is significant when the route of treatment is intravenous and insignificant when the route is intraperitoneal. The results are of the same magnitude for

TABLE 6.—Graft versus host reaction (G.V.H.R)*

Compound	Mg/kg	A†	B‡	C§	D¶	E**
DNR	2.50 iv	2.91	4.65 (S)	3.32	7.93	11.45
DOXO	1.25 iv	2.91	3.02	3.32	7.93	9.00
DNR	0.75 ip	3.23	4.05 (S)	2.94	10.25	6.31 (S)
DOXO	0.75 ip	3.01	4.43 (S)	2.96	8.08	5.45 (S)

*$C_{57}B1$/Rho Ico male mice → $B_6D_2F_1$/Ico male mice.
Day 0: 10^8 spleen cells (iv route).
Treatments (iv route) on Days 1, 5, and 9 or (ip route) on Days 1, 2, 5, 6, 7, 8, and 9.

$$\text{On Day 14: splenic index} = \frac{\text{spleen weight (mg)}}{\text{mouse weight (g)}}.$$

†A = controls
‡B = compound controls
§C = isolog controls
¶D = G.V.H.R.
**E = G.V.H.R. grafted mice are treated
S = significant ($P = 0.05$)

doxorubicin (table 7). We have not, therefore, been able to confirm the results of Vecchi et al (66), who claim a difference between the immunodepressive activity of daunorubicin and doxorubicin.

Mutagenic Activity

Chromosome damage, principally chromatin breakage, produced by daunorubicin has been noted for many years (4,68–73). It is found directly in the bone marrow or blood cells or in cultured cells; anomalies may also be found in oral epithelial cells (74). The type of chromosome change depends upon the experimental animal and is at the highest level 24 hours after treatment (75,76). Usually, there is a long-term persistence of genetic damage induced by chemotherapy (77). In human leucocyte cultures, chromosome aberrations induced by daunorubicin are synergistically heightened by arginine (78). Daunorubicin, like doxorubicin, is a potent inducer of sister chromatid exchange (SCE) (79,80). The cytogenetic effect of carminomycin is more pronounced than is that of daunorubicin (81). Using different microbiological systems, Marquardt and Marquardt (82) found that daunorubicin is an active mutagen. With the Ames test (strain TA 98), we found that daunorubicin is a slightly more potent mutagen than doxorubicin (table 8).

Teratogenic Activity

The teratogenic activity of daunorubicin was studied in the rat (83,84). Daunorubicin is less teratogenic than doxorubicin on a mg/kg basis when given ip to rats. The rabbit is less sensitive than the rat to the teratogenic activity of daunorubicin. Neither drug was teratogenic when given iv to the rabbit, but a high incidence of abortion occurred (85,86). Dike (87) also showed this teratogenic activity in the rat, but not in the rabbit. Daunorubicin may cause azoospermia, usually reversible.

We did not find teratogenic effects in the chick embryo or in the mouse, but we did find embryotoxic effects. In the rabbit (iv treatments), the teratogenic activity was weak. When given to an adult female dur-

TABLE 7.—In vivo tumor immunity (Ehrlich tumor cells)*

	Mg/kg	Route	R†	Results‡
Daunorubicin	0.75	ip	1.6	NA
Doxorubicin	0.75	ip	1.3	NA
Cyclophosphamide	25.00	ip	3.3	A
Daunorubicin	5.00	iv	2.2	A
Doxorubicin	2.50	iv	2.0	A

*OF$_1$ female mice, 6 to 8 weeks old.
Immunization on Days -19, -13, and -7.
8 treatments on Days -20, -18, -17, -14, -12, -10, -6, and -4.
Challenge on Day 0 (5 x 10^4 malignant Ehrlich cells).
Results on Day 80.

$$^{\dagger}R = \text{mean of survival time } \frac{\text{immunized mice}}{\text{immunized and treated mice}}$$

†The higher the value is for R, the more depressive is the compound.
‡A = depressive activity; NA = no depressive activity.

TABLE 8.—Mutagenic activity of daunorubicin*

	Number of revertants nmoles/plate				
Compound	50	20	10	1	0.5
Daunorubicin	Bactericide	406	335	11	2
Doxorubicin	Bactericide	378	172	6	3
Daunorubicin	Bactericide	91	52	6	0
Doxorubicin	150	57	19	0	0

*B. N. Ames test [Proc Nat Acad Sci 73: 950, 1976. S.] typhimurium TA 98 his—("frameshift mutation"). Direct method (without S-9).

ing pregnancy, daunorubicin may produce embryopathy (88), but delivery of a normal child after chemotherapy of acute promyelocytic leukemia during pregnancy has been reported (89). Nevertheless, daunorubicin should not be given during the first 3 months of pregnancy; later, the danger of malformation is lessened, but it still exists.

Carcinogenic Activity

Different types of tumors are induced in Sprague-Dawley female rats after iv doses of daunorubicin: mammary, hepatic, renal, and uterovaginal (90,91). This carcinogenic potential was confirmed in the rat by Bertazzoli et al (92) who observed mammary adenocarcinoma with daunorubicin and fibroadenoma with doxorubicin. Other studies confirm the carcinogenic activity: Solcia et al (93), Bucciarelli et al (94), Philips et al (95), and Weisburger et al (96).

Mutagenesis and malignant transformation in cell cultures are induced by daunorubicin or doxorubicin (82,97). In the mouse, we found the induction of fibrosarcoma with daunorubicin.

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Risk Factors for Development of Daunorubicin Cardiotoxicity

Daniel D. Von Hoff,[1,*] and Maxwell Layard[2]

SUMMARY

Daunorubicin has been in clinical trials in the United States for the past 15 years. In 1966, Dr. David Karnofsky was the first investigator to report congestive heart failure in patients who had received high doses of daunorubicin (1). This early report was followed by a study by Halazun et al (2), colleagues who carried out a randomized controlled trial of vincristine plus prednisone versus vincristine plus prednisone plus daunorubicin. They reported a 0% incidence of congestive heart failure in the two-drug regimen and a 9.99% incidence in the three-drug regimen containing daunorubicin. Since that report there have been a large number of reports in the literature documenting that congestive heart failure can occur with administration of daunorubicin (3–19). Unfortunately, even though the drug-induced cardiotoxicity is a well-documented phenomenon, there has been very little information available as regards its incidence or risk factors for its development. This is in contrast to Adriamycin, where both the incidence of and risk factors for the development of cardiotoxicity are better studied. Because of this paucity of information we began a large retrospective study in 1976 to determine the incidence of and risk factors for the development of daunorubicin-induced cardiotoxicity. The incidence data have been reported previously (20). This communication will review the incidence data and report new findings on the risk factors.

[Cancer Treat Rep 65 (Suppl 4):19–23, 1981]

MATERIALS AND METHODS

This analysis included 5613 patients who had received daunorubicin in cooperative group studies (4236 patients) or from independent investigators (1377 patients). Most of these patients had acute lymphocytic or acute myelocytic leukemia. About one half of the patients had received the drug in combination with other active antileukemia agents. Flow sheets from each of these patients were examined and any cardiotoxicity felt to be due to daunorubicin by the investigator caring for the patient was recorded as drug-induced cardiotoxicity.

RESULTS

General

Of the 5613 patients studied there were only 110 or 1.96% who developed what the principal investigators thought was daunorubicin-induced cardiotoxicity.

As the analysis proceeded it became clear that like Adriamycin, the clinical cardiotoxicity secondary to daunorubicin could be divided into two separate groups. The first group were patients who had the signs and symptoms of congestive heart failure that was felt by the investigator to be due to daunorubicin. These signs and symptoms usually included unexplained tachycardia which was sometimes commented on for weeks before overt congestive heart failure occurred.

There was a second group of patients who had electrocardiographic changes alone without any of the signs or symptoms of heart failure. In this analysis the cardiomyopathy group was considered first.

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Drug-Induced Congestive Heart Failure

In the entire population there were 65 cases of drug-induced congestive heart failure for an overall incidence of 1.16%. This overall incidence was surprisingly low. This could be because this study only examined investigator-observed, clinically overt congestive heart failure and many of the patients had received only small doses of daunorubicin.

Some of the early literature had suggested that the daunorubicin-induced cardiomyopathy was a total dose-related phenomenon. To see if this was the case, the cumulative percent incidence of congestive heart failure was plotted versus the total dose of daunorubicin in mg/m^2 (fig 1). A curve was fit to the data points and a clear cut dose-response curve was recognizable. In the entire population that received up to and including 550 mg/m^2 of daunorubicin there was only a 4% incidence of congestive heart failure. At a total dose of 1050 mg/m^2 there was a 14% incidence of congestive heart failure. These percentages are lower than one might expect. It is obvious from this graph that there is a continuum of increasing risk with increasing doses of daunorubicin and that there is no absolute cutoff point which should not be exceeded under any circumstance.

Other Risk Factors

In addition to total dose, other possible risk factors were examined. These factors included: length of followup after last dose of daunorubicin, patient age, prior radiotherapy to the mediastinum, prior cardiac disease, performance status, race, and sex. Schedule of drug administration could not be examined because induction and reinduction leukemia regimens were extremely variable.

The first possible risk factor examined was the length of followup after last dose of daunorubicin. In other words, is a patient who has received daunorubicin ever out of danger of developing a cardiomyopathy? The time between the last dose of daunorubicin and the development of congestive heart failure presents a rather broad range of 14–280 days with a mean of 89 and a median of 63 days. This means that one half of patients took greater than 63 days after the last dose of daunorubicin to develop clinical signs and symptoms of congestive heart failure. Therefore, in making a clinical decision as to whether to continue daunorubicin in a particular patient one must remember that the cardiac damage from the last dose may not show up for a number of months.

The next risk factor examined was patient age. As noted in table 1 most patients in this study were in the younger age categories. Only the age categories of less than 15 and 15–40 years of age contained enough patients for analysis. When total dose is taken into consideration, the incidence of congestive heart failure at total doses of greater than 300 mg/m^2 was significantly higher in children than it was for adults from 15–40 years of age. At 550 mg/m^2 the risk is 6% for children and 1.3% for adults while at 1100 mg/m^2 the risk is 16% for children and 6% for adults (fig 2). This

TABLE 1.—Ages of patients receiving daunorubicin in the present study

Age category	No. patients
< 15	2861
15–40	2602
40–60	140
> 60	10

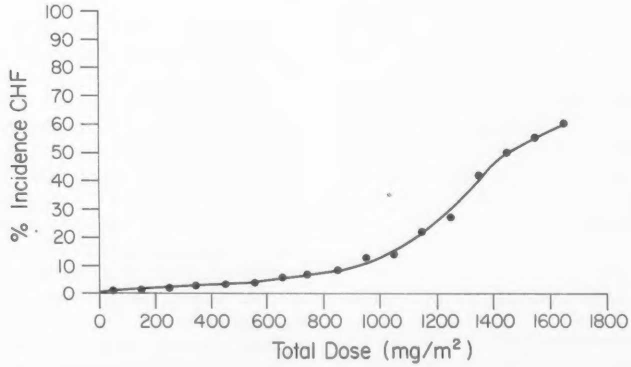

FIGURE 1.—Percent incidence of congestive heart failure (CHF) in patients receiving daunorubicin (5613 patients—65 cases of CHF).

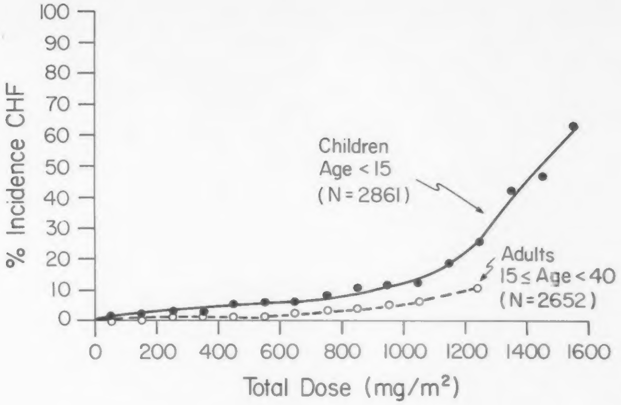

FIGURE 2.—Effect of age on percent incidence of daunorubicin-induced congestive heart failure (CHF).

unexpected finding remains unexplained but is also being noted by other investigators both for daunorubicin and for Adriamycin (21,22). The data for performance status, sex, and race will not be shown but none of these factors indicated an increased risk of developing drug-induced congestive heart failure.

There were two possible risk factors which this study unfortunately could not assess. There were too few patients with prior cardiac disease and previous mediastinal radiotherapy so these two factors could not be evaluated. It is extremely unlikely that the effect of these two factors on the risk of development of daunorubicin-induced congestive heart failure will ever be known since with over 5000 patients we could not answer the question. It should be noted that prior cardiac disease and prior radiotherapy to the mediastinum have been anecdotally implicated in the literature as risk factors for development of daunorubicin-induced congestive heart failure.

Outcome of Congestive Heart Failure

Within an average followup time of 70 days after the onset of congestive heart failure, 63 of the 65 patients were dead. Of the 63 deceased patients, 27 had autopsy examination of the heart. Of these 27 patients, only 10 (37%) had light microscopic myocardial findings reported as compatible with daunorubicin-induced congestive heart failure. One patient had tumor infiltration of the heart and the other 16 hearts were reported as microscopically normal. Electron microscopy was not routinely performed.

This low incidence of pathologic confirmation could mean the clinical diagnosis of drug-induced congestive heart failure is frequently incorrect or that there are difficulties in interpreting the histologic changes secondary to daunorubicin.

Daunorubicin-induced Electrocardiographic Change

As a final consideration, of 5613 patients receiving daunorubicin there was a 0.80% incidence of electrocardiographic (ECG) changes. This low incidence of ECG changes is tempered by the fact that most patients were not monitored during the infusion of daunorubicin and electrocardiograms were performed at random times after the dose of drug. Most changes were nonspecific and have been previously reported (20). An important finding of this study was that ECG changes were not related in any way to total doses of daunorubicin administered and did not presage the development of drug-induced congestive heart failure.

DISCUSSION

Keeping in mind that this was a retrospective study, the following conclusions can be drawn: 1) First of all, there are two patterns of daunorubicin-induced cardiotoxicity: a drug-induced congestive heart failure or cardiomyopathy and drug-induced electrocardiographic changes. 2) The overall incidence of daunorubicin-induced congestive heart failure is 1.16%. 3) The probability of developing daunorubicin-induced congestive heart failure is related to total dose of daunorubicin administered with a continuum of increasing risk. There is no absolute cutoff point which should not be exceeded under any circumstances. Clinical judgment must be used. 4) Additional risk factors for the development of daunorubicin-induced congestive heart disease include: young age < 15 and prolonged followup after the last dose of drug. 5) Finally, daunorubicin-induced congestive heart failure is lethal in 75% of cases.

As far as treatment is concerned, conventional medical management for heart failure has been the rule. One should remember that some investigators feel that patients on daunorubicin have hearts that are extremely sensitive to digitalis. In the literature only about 25% of patients have responded to medical management of their drug-induced heart failure. That figure may be too low.

Given such a low incidence of daunorubicin-induced congestive heart failure, why should an oncologist worry about daunorubicin cardiotoxicity? For one thing, with improved survival times there will be longer followup times and hence a greater chance for drug-induced congestive heart failure to appear. Secondly, 84% of the 65 patients dying with daunorubicin-induced congestive heart failure were in complete remission from their leukemia.

In addition to looking at risk factors for developing daunorubicin-induced congestive heart failure, there may now be ways to monitor patients' cardiac functions to try to predict who will and who will not develop drug-induced congestive heart failure. A great number of monitoring techniques have been advocated for the early detection of anthracycline-induced congestive heart failure, including serial measurement of voltage on electrocardiograms (23-25), cardiac enzymes (26,27), systolic time interval measurements (28-31), QRS-Korotkoff intervals (32,33), and echocardiograms (34-37). More recently transvenous cardiac biopsies (21,22) and radionuclide angiocardiography (23,24) have emerged as promising methods for predicting the clinical development of Adriamycin-induced congestive heart failure. There are as yet no published reports of uses of these latter two techniques on patients receiving daunorubicin. It should be remembered that the clinical usefulness of the first five techniques mentioned is controversial as some investigators feel that by the time changes are evident in these tests the patient already has clinically evident congestive heart failure. Transvenous cardiac biopsies and radionuclide angiocardiography are not available to every oncologist or hematologist using daunorubicin. In summary, at present none of these tests seem clearly indicated for following the routine patient on daunorubicin. This situation may change as more data become available for the radionuclide angiocardiography technique.

A number of agents have been reported which could theoretically help protect a patient from developing anthracycline-induced cardiomyopathy. These include Vitamin E, coenzyme Q10, digoxin, prednisone, and carnitine. None of these agents have been proven effective in controlled randomized trials in man. In addition, the overall incidence of daunorubicin-induced congestive heart failure is so low that any prospective study with and without a protective agent such as vitamin E would require a very large number of patients.

Overall, although it has been known for a number of years that daunorubicin is a cardiotoxic agent, there is still a great deal to be learned about the etiology and prevention of daunorubicin-induced cardiotoxicity.

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Daunorubicin Treatment of Adult Solid Tumors

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In 1963 Di Marco and associates (1) reported the isolation of a new antibiotic, which they named daunomycin, from cultures of the soil microbe *Streptomyces peucetius*. Shortly thereafter, a group of French investigators (2) independently reported isolation of a substance from *Streptomyces coeruleorubidus* that they designated rubidomycin. Subsequently, these two antibiotics were found to be identical, and the name daunorubicin was applied to give credit to both groups of discoverers. The demonstration of activity of daunorubicin in animal tumors (3) led to clinical trials initiated by DiPietro (4) at the Istituto Nazionale Tumori in Milano. Sixteen patients with a variety of tumors were treated intravenously in these initial trials, and responses were noted (one lymphoma, one head and neck cancer, and one melanoma).[4] In addition, some patients with head and neck cancer had tumor remissions after treatment with intra-arterial infusions of daunorubicin (4).

The French investigators also began clinical trials with daunorubicin in the mid-1960s and reported activity in a wide cross-section of cancers (5–9). Although responses were seen in patients with solid tumors, interest in the drug was particularly generated by the fact that it produced complete remissions in acute myelogenous leukemia (9,10).

Clinical trials were begun at one American institution at the same time as those in Europe were being conducted. Tan and associates (11) tested daunorubicin in a variety of tumors in adults and children with a special focus on acute leukemia. Only 14 adults were treated in this series, but two tumor responses were seen (11).

Since these small early studies with daunorubicin in the 1960s, there have been very few trials in adult solid tumors, and very few of those that have been done have been adequate trials. There appear to be three reasons for this lack of study. First, the clinical orientation of many of the original investigators was to the treatment of only leukemias and lymphomas. Second, in 1968, doxorubicin (Adriamycin), a daunorubicin analog, was discovered by Italian investigators and was found more effective than daunorubicin in a number of experimental animal tumors (12). When doxorubicin was first used in clinical trials at the Istituto Nazionale Tumori, it created a great deal of excitement because it produced prompt responses in a wide range of tumors at doses which did not cause inordinate toxic effects (13). Further testing of daunorubicin in solid tumors was essentially abandoned in favor of testing this newer, and possibly more effective, anthracycline. Third, the major toxic effect of daunorubicin is myelosuppression that can be severe and prolonged. Normal bone marrow stem cells seem to be very sensitive to daunorubicin, and there is a narrow toxic-therapeutic ratio. Because rapid production of marrow hypoplasia is the therapeutic goal in acute myelogenous leukemia, the myelosuppressive effect of daunorubicin makes it a useful drug in this disease, but it is much less useful in other forms of cancer.

We have reviewed the published and unpublished American and European experience with this drug in adult solid tumors. The data are sparse and consist mostly of small series of patients representing many tumor types, and in some reports the criteria of response are not the rigid ones used in current clinical trials. The patients treated with daunorubicin who were reported to the NCI, but whose results were never published, came from a variety of sources. Some represented a series of as few as two to three cases. Others, numbering several dozen cases, came from studies of three cooperative groups, namely, the Western Cancer Study Group, the Southeastern Cancer Study Group, and the Acute Leukemia Group B. A variety of doses and schedules, listed in table 1, were used in these trials. There seems to be little relation between dose-schedule and response, but this point has not been adequately studied. Within the constraints of general scarcity and uneven quality, we will discuss and enumerate the available data.

COLON CARCINOMA

Metastatic colon carcinoma is one of the few tumors in which daunorubicin has had meaningful testing (table 2). Weiss and Cantor (14) treated 57 patients, all

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[4] Unpublished data in files of the Investigational Drug Branch, Division of Cancer Treatment, National Cancer Institute.

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TABLE 1.—Doses and schedules of daunorubicin used in published and unpublished clinical trials in adult solid tumors

1. 1 mg/kg every 4th day × 4 doses
2. 0.8 mg/kg initially, then 0.6 mg/kg weekly
3. 30 mg/m^2 daily × 5
4. 45 mg/m^2 BIW × 5 doses
5. 80 mg/m^2 weekly
6. 40 mg/m^2 × 5 days
7. 0.75 mg/kg × 4 days
8. 1 mg/kg daily until toxicity or tumor response
9. 30-50 mg/m^2 daily × 5, rest 5 days and repeat
10. 0.8 mg/kg daily × 4, rest 5-10 days; then 0.5-0.8 mg/kg × 1-2 days until toxicity

TABLE 2.—Results of daunorubicin treatment in metastatic colon carcinoma

Treatment (reference)		Responses/total patients
Single agent therapy		
Weiss et al (14)		6 PR/57 (10.5%)
National Cancer Institute data summary		0/12
	Total	6/69
Combination therapy		
White et al (16) Daunorubicin 40 mg/m^2 Day 1 & 29 Methyl CCNU 150 mg/m^2 Day 1 5-FU 350 mg/m^2 weekly		6/38 (16%)

of whom had tumors resistant to 5-fluorouracil (5-FU). The dose used was 0.4 mg/kg on Days 1–3 and 8–10, then 0.4 mg/kg weekly. There were six partial remissions (10.5%), which is unusual because patients with 5-FU-resistant colon carcinoma rarely respond to such second-line therapy. Only four percent of the patients had leucopenia below 2000 cells/mm^3 and none had thrombocytopenia below 50,000 platelets/mm^3. In a similar study by Frytak et al (15) with doxorubicin, only one of 32 previously treated patients with colon cancer responded. The treatment result of Weiss and Cantor (14) has not been confirmed, but, if it were, daunorubicin might be interesting to test in a combination regimen. In unpublished data at the National Cancer Institute (NCI) there were no responses in 12 patients.

Only one combination regimen using daunorubicin has been tried in metastatic colon carcinoma. A study by the Cancer and Leukemia Group B incorporated daunorubicin into a three-drug combination with methyl CCNU and 5-FU (16). Among 38 previously untreated patients, there were five partial remissions and one complete remission, for a total response rate of 16%. The authors stated that the 16% response rate was no better than that usually achieved with 5-FU alone and the regimen was abandoned. Before daunorubicin is used in any other combination regimen in colorectal cancer, it would be best to confirm its activity in another disease-oriented phase II study.

LYMPHOMAS

Among the initial reports of daunorubicin use in the French literature were two studies of non-Hodgkin's lymphoma (5,6). Ten patients were treated and six had responses, although the degree of remission was poorly defined. These responses and those achieved in acute leukemia encouraged other French investigators to test daunorubicin in combination with vincristine and prednisone for lymphomas (17,18). Although some of the treated patients may have had conditions that we would more accurately define today as lymphocytic leukemia, among 18 patients there were five complete remissions (table 3).

French investigators also used daunorubicin in advanced Hodgkin's disease patients, all of whom had been previously treated (table 3) (8). Of 20 patients, one had a complete response and four had partial remissions. A total of 13 patients have been reported to the NCI; three had partial remissions.

BLADDER CARCINOMA

An unusual route of administration of daunorubicin is intravesical instillation. One Italian investigator (19,20) has used daunorubicin in this manner in a few cases of primary papillary bladder tumors and stated that the bladder mucosa tolerated the drug well with no cystitis. There seemed to be little or no absorption of the drug because myelosuppression did not occur, and, in those patients whose daunorubicin blood levels were tested, no drug could be detected. Of four patients treated intravesically, two had partial shrinkage of the primary tumor. Seven patients had intravesicular instillation "prophylactically" after surgical removal of the tumor. Only one patient had a recurrence, but it is difficult to assess how much of a role the drug treatment played in the results. No one else has used this route of administration. No other patients with bladder cancer in any stage have been reported to have been treated with daunorubicin.

HEAD AND NECK CANCERS

Italian investigators (21) have used daunorubicin intra-arterially for treatment of various squamous head and neck cancers. Seven patients received

140–280 mg daunorubicin by infusion in the external carotid artery. Two of these patients had minor responses. The investigators later used daunorubicin in combination with methotrexate intra-arterially and achieved a better response rate (three partial remissions and three minor responses in 10 patients). However, it is not possible to discern what contribution daunorubicin made to this result.

MISCELLANEOUS CANCERS

Table 4 lists the results of daunorubicin treatment in a variety of cancers. It is apparent that, with the exception of soft tissue sarcomas, the number of patients studied is small. This is particularly true of some of the common cancers such as gastrointestinal and breast.

TABLE 3.—Results of daunorubicin treatment in lymphomas

	Responses/no. of patients			
Tumor type and treatment	NCI data	Other data	(ref)	Total
Non-Hodgkin's (all histologic types)				
Single agent	1/22	1/1	(11)	
		5/7	(6)	8/33—24%
		1/3	(5)	
Combination drug therapy				
Daunorubicin 60 mg/m²				
Vincristine 2 mg/m²		9/12	(17)*	9/12—75%
Prednisone 100 mg/m²				
Daunorubicin 20 mg/m² × 2 days, repeat 1 wk				
Vincristine 0.1 mg/kg weekly × 2		2/6	(18)†	2/6—33%
Corticosteroid 100 mg/m²/day				
Hodgkin's disease	3/13	0/1	(11)	8/34—23%
		5/20	(8)‡	

*Includes several children; responses included 4 CR.
†Leukemic phase of lymphosarcoma; responses included 1 CR.
‡Responses included 1 CR.

TABLE 4.—Results of daunorubicin treatment in miscellaneous cancers

	Responses/no. of patients			
Tumor type	NCI data	Other data	(ref)	Total
Breast	0/2	—	—	0/2
Lung	1/13	1/11	(22)	2/24— 8%
Soft-tissue sarcoma	8/53	0/4	(11)	8/57—14%
Bone sarcoma	0/12	0/1	(11)	0/13
Kaposi's sarcoma		0/2	(23)	0/2
Melanoma	2/9	0/3	(11)	
		0/3	(5)	2/15—13%
Mesothelioma	0/3	—	—	0/3
Myeloma	0/2	—	—	0/2
Prostate		1/2	(20)	1/2
Ovarian	0/5	0/1	(11)	0/6
Gestational choriocarcinoma	0/4	—	—	0/4
Renal	1/11	—	—	1/11
Testicle	0/11	—	—	0/11
Gastric	0/4	—	—	0/4
Pancreatic	0/1	0/1	(5)	0/2
Adenocarcinoma from unknown primary	1/10	—	—	1/10
Salivary gland	0/3	1/1	(24)	1/4

CONCLUSION

Daunorubicin has had inadequate trial in all forms of cancer except acute leukemia. The only two cancers represented by more than one or two dozen patients are colon carcionoma and soft-tissue sarcomas. For colon carcinoma there has been only one phase II disease-oriented trial, and in it there was a 10.5% response rate. This regression rate may be of some interest since all patients were resistant to 5-FU. Perhaps daunorubicin should be investigated further in colon cancer. The response results for soft-tissue sarcomas are unpublished and cannot be fully evaluated by others. A response rate of 14% is not very exciting in comparison with the higher ones usually achieved with doxorubicin. However, the number of patients studied has not been adequate to make a final statement about daunorubicin activity in sarcomas.

Partial activity has been noted in lymphomas, but, again, there has not been an adequate study. Doxorubicin is also active in lymphomas and may be superior to daunorubicin, but there are no data to support this.

Only one study has ever been done to compare daunorubicin with doxorubicin. Evans et al (25) did a randomized comparison of the two drugs in childhood solid tumors and found no difference in the response rate in neuroblastoma, one of the childhood cancers responsive to daunorubicin. The myelosuppressive action of daunorubicin, which is so useful in the treatment of acute leukemia, creates a disadvantage in treating solid tumors. Thus, although no good phase II study of daunorubicin exists in any cancer, it might be difficult to incite any interest in initiating either disease-oriented phase II studies or randomized comparisons of the two anthracyclines.

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Daunorubicin-Prednisone in the Treatment of Acute Nonlymphocytic Leukemia[1]

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SUMMARY

In 1967 we first started to use daunorubicin and prednisone in the treatment of acute nonlymphocytic leukemia. In this review we describe our results with this regimen in 72 adults who were treated at the University of Minnesota between 1967 and 1977. Fifty-five patients were previously untreated and 17 had received prior cytotoxic chemotherapy. Complete remissions were obtained in 21 (29%) of the 72 patients. The patients achieving complete remission were significantly younger and had lower pretreatment leukocyte and absolute myeloblast counts than the nonresponders. Patients with acute promyelocytic leukemia had a higher response rate (67%) and longer duration of complete remission compared to patients with the morphologic subtypes. Their median duration of complete remission was 29 months compared to 6 months for the other patients. The 21 patients who achieved complete remission survived longer (20 months) than those who failed to enter remission (2 months). Our experience suggests that daunorubicin is an effective drug in the treatment of acute promyelocytic leukemia, but should be combined with additional agents in the treatment of other types of acute nonlymphocytic leukemia.

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Since the completion of initial clinical trials, daunorubicin has been recognized as the most active single agent for the remission induction therapy of patients with acute nonlymphocytic leukemia (1–10). Most of the cytotoxic drugs which were in use prior to the time daunorubicin became available had significant activity in the treatment of acute lymphoblastic leukemia, but were relatively ineffective in patients with acute nonlymphocytic leukemia. In acute nonlymphocytic leukemia, these other drugs resulted in complete remission rates of less than 25% compared to 20%–50% for daunorubicin alone (1–10). More recently, anthracycline antibiotics related to daunorubicin have also been found to be very effective when used alone (11,12). However, the combination of the anthracyclines with other active drugs has resulted in induction regimens which have produced much higher remission rates and longer survival than daunorubicin alone (13–18). This review summarizes our past experience with daunorubicin and prednisone and confirms the need to employ daunorubicin in combination with other effective chemotherapeutic agents.

MATERIALS AND METHODS

From 1967 through 1977, 72 adults with acute nonlymphocytic leukemia were treated with daunorubicin and prednisone at the Masonic Leukemia Treatment Center of the University of Minnesota. There were 43 males and 29 females in the study population, and they ranged in age from 15 to 80 (median, 49) years. Twenty-one patients (29%) were ≥ 60 years of age. The diagnosis and subclassification of acute nonlymphocytic leukemia were based on the original pretreatment bone marrow samples using criteria and methods previously described (19,20).

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All patients covered by this report were treated with a regimen that included daunorubicin (1 mg/kg/day) administered by rapid iv infusion and prednisone (20 mg three times daily) orally for 5 successive days (1). The prednisone dose was then tapered to 40 mg/day until the platelet count had risen to > 100.0 × 10^9/l. Repeat courses of treatment, when necessary, were usually begun on Day 21. All patients treated at the Masonic Leukemia Treatment Center between 1967 and July 1973 received daunorubicin and prednisone. From July 1973 through December 1974, patients were randomly assigned to receive either daunorubicin and prednisone or a regimen of cytosine arabinoside and 6-thioguanine (21). Only those with the diagnosis of acute promyelocytic leukemia were treated with daunorubicin and prednisone from 1975 through 1977. All other patients with acute nonlymphocytic leukemia were treated with the combination of daunorubicin and cytosine arabinoside during this latter period (18).

Patients were treated in private rooms on the regular ward and were placed in protective isolation when their leukocyte count fell to less than 1.0 × 10^9/l. Packed rbc transfusions were administered to maintain the hemoglobin above 9.0 g/dl and prophylactic platelet transfusions were generally administered three times a week until the platelet count rose above 30.0 × 10^9/l. In recent years, daily platelet transfusions have been used. Prior to July 1971, most patients received prophylactic antibiotics including oral lincomycin and neomycin. When an infection developed, appropriate antibiotic treatment was instituted. Since July 1971, treatment with broad spectrum antibiotics including cephalothin, gentamicin, and carbenicillin has been initiated empirically in all patients when body temperature rises to > 38.4 °C (22).

Patients with acute promyelocytic leukemia received additional treatment specifically for coagulopathy (20). Heparin sodium (10 units/kg/hour) was generally administered as a continuous infusion from the time of diagnosis. The infusion of heparin was adjusted to maintain the thrombin time between 20 and 60 seconds at a 1:2 dilution of the patient's plasma in normal plasma. All patients continued to receive heparin until a slowing of the disseminated intravascular coagulation was documented. Cryoprecipitate was transfused to maintain the plasma fibrinogen level > 50%. Vitamin K (25 mg/day iv for 3 days and 10 mg iv daily thereafter) was given as long as the patient was receiving antibiotics. Platelet transfusions were administered daily.

Maintenance chemotherapy was started upon documentation of complete remission. Early in the study, maintenance chemotherapy consisted of hydroxyurea (80 mg/kg orally twice weekly) in 12 patients (1) and either 6-mercaptopurine or daunorubicin as single agents in two patients. Seven patients received 6-thioguanine (2 mg/kg orally) for 4 successive days each week followed on Day 5 by cytosine arabinoside (1.5 mg/kg im) (23).

A complete remission was defined using the criteria of the CALGB (24). The time to complete remission was measured from the initiation of therapy to the first post-treatment marrow test results documenting that remission. The duration of remission was measured from the date of this bone marrow test to the first demonstrable evidence of recurrent leukemia. Since some of the patients had previously received treatment, survival was calculated from the initiation of chemotherapy with daunorubicin and prednisone. Differences in the clinical features among groups of patients were evaluated by means of the Pearson chi-square test for percentage differences and the Mann-Whitney rank test for differences in continuous variables. Differences in survival and the duration of remission between groups were evaluated by the Mantel-Haenzel one-degree-of-freedom chi-square test (25).

RESULTS

Pretreatment Clinical and Laboratory Data

Seventy-two adults with acute nonlymphocytic leukemia were treated with daunorubicin and prednisone. Fifty-five patients were previously untreated and 17 had received prior cytotoxic chemotherapy. This included vincristine or 6-mercaptopurine alone or in combination with prednisone in seven patients and combination therapy, such as the VAMP regimen, in ten. No patient had previously received daunorubicin; only one had received doxorubicin.

The 72 patients were distributed among four subtypes of acute nonlymphocytic leukemia. Forty-four patients were classified as having acute myelogenous leukemia, eight acute myelomonocytic, nine acute promyelocytic, and 11 erythroleukemia. The median pretreatment hematologic values were: hemoglobin, 8.6 g/dl; wbc, 8.0 × 10^9/l; and platelet count, 45.0 × 10^9/l. The median percentages of myeloblasts in the blood and bone marrow were 46% and 67%, respectively.

Induction of Remission

Complete remissions were obtained in 21 (29%) of the 72 patients undergoing chemotherapy. Nine patients did not complete a full course of chemotherapy, but they are still included in the results. Eighteen of the patients entered complete remission with only one course of chemotherapy, and three patients required two courses. The median time to complete remission was 26 (range, 14–79) days.

A variety of factors were evaluated to determine their effect on response to therapy. The effect of age was most striking. The median age of those achieving complete remission was 39 years compared to 50 years for those failing to enter complete remission ($P < 0.05$). Nineteen (38%) of 51 patients < 60 years of age achieved complete remission compared to only two (10%) of 21 patients ≥ 60 years of age ($P < 0.05$). Other patient characteristics such as gender or exposure to prior cytotoxic chemotherapy did not significantly affect the response to daunorubicin and prednisone. However, patients with acute promyelocytic leukemia had a significantly higher complete remission rate (67%) than all other types of acute nonlymphocytic leukemia combined (24%) ($P < 0.025$).

Certain pretreatment hematologic features were evident in the responders. They had a median leukocyte count of 3.1×10^9/l compared to 17.2×10^9/l for the nonresponders ($P < 0.05$). In addition, the absolute myeloblast count in the peripheral blood was also lower in the patients destined to enter complete remission (2.9×10^9/l) than in those destined to fail therapy (13.3×10^9/l) ($P < 0.01$). The median hemoglobin, platelet count, and myeloblast percentage in the bone marrow were similar in both groups.

Remission Duration

The median duration of complete remission for the 21 responders was 7 (range 2–68+) months (fig 1). Nineteen patients relapsed after 2–41 months in complete remission. However, two patients currently remain in their initial remissions at 49 and 68 months, respectively. Both patients remaining in remission have acute promyelocytic leukemia. The median duration of complete remission for the six patients with acute promyelocytic leukemia is 29 months compared to 6 months for the other morphologic subtypes combined ($P < 0.007$). However, the influence of histologic subtype on remission duration cannot be adequately separated from that of the maintenance chemotherapy in this study, since all of the patients with acute promyelocytic leukemia received cytosine arabinoside plus 6-thioguanine while only one patient with another subtype of leukemia received this maintenance regimen.

Survival

Median survival from the initiation of therapy with daunorubicin and prednisone was 4 months for the entire group of 72 patients and ranged from < 1 to ≥ 104 months (fig 2). Response to the induction therapy was a major determinant of survival. For the 51 patients who failed to respond, the median survival was only 2 months. Of these patients, only six survived 12 months or longer. However, one patient who failed to respond to daunorubicin and prednisone subsequently achieved a complete remission with another regimen and is alive at 104 months. The median survival for the 21 patients who entered complete remission was 20 months. Eight patients survived for > 2 years and four for > 3 years. Currently, two patients remain alive at 50 and 69 months.

Toxicity

Twenty-three patients died within 30 days of receiving daunorubicin and prednisone. These deaths during the induction period were attributed primarily to sepsis (nine), hemorrhage (five), and both sepsis and hemorrhage (seven). Two patients died of other causes. Daunorubicin cardiotoxicity was not a cause of death in this study. The median age of the patients who died during the induction period was 54 (range, 19–80) years. Ten were > 60 years of age. Thus, 48% of all patients treated with daunorubicin and prednisone

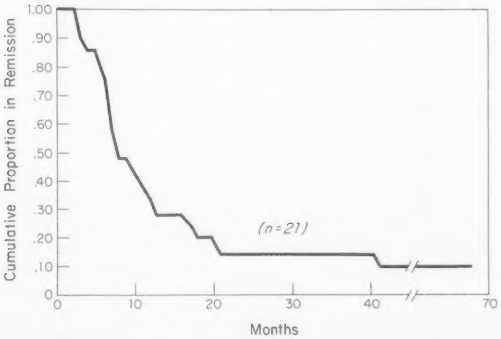

FIGURE 1.—Duration of complete remission in patients undergoing induction chemotherapy with daunorubicin and prednisone.

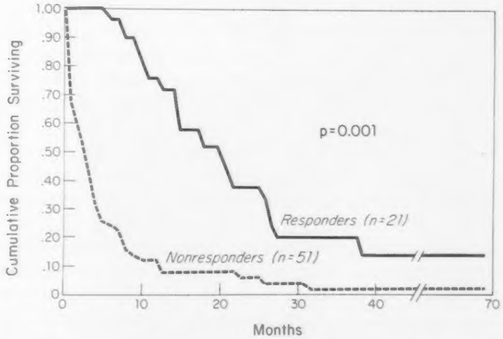

FIGURE 2.—Survival of responders and nonresponders from the initiation of therapy with daunorubicin and prednisone.

who were over 60 years of age died during the induction period.

DISCUSSION

Major improvements in the treatment of acute nonlymphocytic leukemia have been made over the past decade. However, prior to the introduction of daunorubicin, single-agent chemotherapy produced complete remissions in less than 25% of patients and combination chemotherapy usually resulted in complete remission rates of < 50% (9,21,26). The use of daunorubicin as a single agent in previously untreated patients resulted in complete remissions in 20%–50% (1–3,5,6,8–10), and its subsequent incorporation into intensive chemotherapeutic regimens has resulted in 63%–85% of patients achieving remission (13,14,16–18).

A variety of dosage schedules for daunorubicin have been used in the treatment of acute nonlymphocytic leukemia, but the higher response rates have only been observed with successive daily dose schedules (2,8–10). In a randomized study of 131 previously untreated adults, Weil et al (8) demonstrated that daunorubicin (60 mg/m^2) repeated on 5 successive days resulted in 43% complete remissions compared to only 17%–18% for the same dose given less frequently. High doses of daunorubicin given as a single bolus have also not been as effective as repetitive lower daily doses (5,27). In our study, daunorubicin was given on 5 successive days accompanied by prednisone, and complete remissions were obtained in 29% of the patients treated. The prednisone was employed to protect against the potential risks associated with thrombocytopenia and because prednisone has known activity against acute nonlymphocytic leukemia (28). However, the complete response rate in our patients is within the range of those reported previously for daunorubicin alone; this suggests that the addition of prednisone did not increase the percentage of complete remissions.

Advancing age has been considered an adverse prognostic factor for patients with acute nonlymphocytic leukemia. This was confirmed in our study with daunorubicin and prednisone where the complete remission rate was only 10% in patients > 60 years of age and 38% in those patients < 60. Nearly one half of our patients > 60 years of age died of infection and/or hemorrhage during the induction period. However, by utilizing more effective treatment programs elderly patients may regularly achieve complete remissions (29). Improvements in supportive care have also contributed to higher response rates in the elderly. With the current intensive combination chemotherapy regimens which rapidly produce a high percentage of complete remissions, similar rates of response are observed in patients both > and < 60 years of age (13,15,16). Elderly patients now benefit from intensive induction chemotherapy in a manner comparable to that of younger patients.

Daunorubicin was found effective in the treatment of two morphologic subtypes of acute nonlymphocytic leukemia which had previously been especially difficult to treat successfully. Erythroleukemia was thought particularly resistant to chemotherapy until it was shown to respond to daunorubicin (30). Acute promyelocytic leukemia also responds well to regimens including daunorubicin. In 1973, Bernard et al (31) reported a complete remission rate of 11% in 36 patients treated with regimens not including daunorubicin. With the addition of daunorubicin, 19 (49%) of 39 patients achieved remission. Subsequently, the importance of heparin and blood clotting factor replacement in the management of the coagulopathy associated with acute promyelocytic leukemia was also recognized (20,32). The use of heparin along with chemotherapeutic regimens which include daunorubicin has decreased the incidence of fatal hemorrhage during induction and increased the rate of complete remission (20,32). We treated a total of nine patients with acute promyelocytic leukemia with daunorubicin and prednisone plus concomitant heparin and clotting factor replacement. Six of the nine patients achieved a complete remission. This response rate is significantly higher than that observed in our other 63 patients. In addition, the median duration of complete remission at 29 months for these six patients is considerably longer. These data suggest that daunorubicin is an important drug in the management of patients with acute promyelocytic leukemia. However, daunorubicin, alone or with prednisone, is not sufficient therapy for the other subtypes of acute leukemia.

CONCLUSION

This review of our past experience with daunorubicin and prednisone verifies the efficacy of these drugs in the treatment of adults with acute nonlymphocytic leukemia. It also demonstrates again that patients achieving complete remission have improved survival. The recent utilization of more intensive and more effective combinations has resulted in further improvements both in response rates and survival. Preliminary reports have now appeared documenting long-term survival in 6%–16% of adults with acute nonlymphocytic leukemia treated with intensive chemotherapy (33–36). In this study with daunorubicin and prednisone, two of the patients entering complete remission have remained alive and in remission for > 4 years. These two patients represent less than 3% of our entire study population and emphasize again the need to employ daunorubicin in combination with other effective cytotoxic agents for the treatment of acute nonlymphocytic leukemia.

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Future Clinical Investigations With Anthracycline Antibiotics in Relation to Daunorubicin[1]

Franco M. Muggia,* Ronald H. Blum, and James C. Wernz[2]

SUMMARY

Future studies with daunorubicin involve mainly comparative evaluations with other anthracycline antibiotics and related compounds. Efforts are ongoing to improve the therapeutic index of these drugs. This includes not only laboratory development but also clinical evaluation of analogs, manipulations in dose schedules, and the use of protectors of toxic manifestations, primarily of cardiomyopathy. Technological advances in the clinical area permit, for example, the exploration of daunorubicin and related compounds by continuous infusion under more precise pharmacologic and cardiologic monitoring than was possible when they were initially introduced.

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The role of daunorubicin in induction regimens used in patients with acute myeloblastic leukemia is well established. The drug is also often employed in the treatment of other leukemias. It does not, however, play a prominent role in any other area of therapeutics. On the other hand, the future is bright for continued clinical investigations with doxorubicin (Adriamycin) and other second-generation anthracyclines. It is likely, therefore, that the frontiers of clinical investigation will be relegated to other anthracyclines and not to daunorubicin (DNR). We shall review those considerations which guide the search for new anthracyclines and their future clinical role.

CONSIDERATIONS IN ANALOG DEVELOPMENT

Major aspects of interest in the development of second-generation compounds related to active anticancer agents are the search for totally new selectivity, improvements in therapeutic index either by reducing specific dose-limiting or other toxic effects or by increasing efficacy, or modifications resulting in more favorable pharmacologic features. Any one of these improvements must be carried out at a reasonable cost and result in a compound that is practical for clinical use. These latter considerations, albeit secondary to the former, may be of considerable importance when the advantages of the analog over the parent are not impressive.

Specifically referring to the anthracycline antibiotics, it is worthwhile to compare the toxic effects of the first two drugs that were introduced: DNR and doxorubicin. Their acute and chronic toxic effects are practically identical. Retrospective reviews relative to the risk of developing cardiotoxicity indicates very similar risks in relation to courses of drug administered (1,2). As for its efficacy relative to Adriamycin, the evidence is quite fragmentary (3); however, it is not encouraging to further development of DNR.

With the exception of its current indication for the treatment of acute leukemia of the nonlymphocytic type, and for all refractory or high risk childhood leukemias, little information is available on the efficacy of DNR. Comparative studies against doxorubicin in pediatric solid tumors had been initiated but curtailed because of interruptions in drug availability. Nevertheless, activity was documented against neuroblastoma (3). Trials done in Spain of DNR in combination with other drugs for malignant lymphomas indicate that it is a useful agent for the treatment of these malignancies (J. Vincente, unpublished). However, again, comparative data with doxorubicin

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are lacking. In the more common solid tumors, there is, unfortunately, no information on the potential of this drug.

Experience with DNR and doxorubicin has stimulated the development of new anthracyclines and related drugs that are in several stages of study. Classification into three major groups will help identify the various derivatives and the directions of study.

Closely Related Structural Analogs of DNR and Doxorubicin

The first such compound extensively studied in the clinic was rubidazone which can be considered a prodrug of DNR (4). The evidence for a better therapeutic index of this drug over DNR in leukemia (5) is questionable in the absence of comparative trials, and its activity in solid tumors has been disappointing (6,7). Other closely related analogs such as 4′-demethoxydaunorubicin are just beginning trial. This derivative has enhanced potency and may be active orally. Because of a moderate improvement in therapeutic index against murine tumors as compared to doxorubicin, the 4′-epi-derivative has been undergoing clinical testing (8). Comparative trials in man will likely be required to establish with certainty an advantage over the parent.

Major Modifications in the Anthracycline Aglycone

Carminomycin and aclacinomycin A represent a group of related drugs with demonstrably different biological properties. For example, widely different patterns of drug interaction with DNA and effects on macromolecular synthesis have been described for these compounds relative to DNR and doxorubicin (9,10). Studies concerning the toxic effects of the aglycone by itself have indicated considerable difference between aclacinomycin A's nucleus, aklavinone, and that of doxorubicin and DNR (11). Using acute effects on the electrocardiogram of golden hamsters, no alterations were seen even after 100 mg/kg of aklavinone, whereas all animals experienced changes at less than one third above dosages of adriamycinone and one tenth of DNR (12). Clinical studies will need to explore not only changes in therapeutic index but also the question of new selectivity and the possible lack of cross-resistance.

Miscellaneous Modifications

These include the newly introduced AD-32 that represents a major change in the pharmacology and lipid solubility of the anthracyclines (12,13). In studies, both in man and non-human primates, AD-32's extensive metabolism has been characterized and a much more rapid biliary excretion than doxorubicin identified (13). At 24 hours the total fluorescence excreted in bile of cynomolgus monkeys was 24.5% after doxorubicin contrasted with 30.25% after AD-32; an inverse relationship was determined in urine (13). Moreover, the decay of AD-32 in plasma shows a much shorter half-time of the slow phase contrasted with doxorubicin. This indicates reduced tissue binding in spite of greater lipophyllicity (13). Since AD-32 needs to be administered in a 24-hour infusion, comparative clinical studies with anthracyclines administered in an analogous way must be carried out in the future. Preliminary reports of doxorubicin administered by infusion have been encouraging (14), and the studies need to be extended to include other drugs including DNR. The technology for the administration of chemotherapeutic agents by infusion has made considerable strides recently (15).

Novel attempts at modifying the therapeutic index have been made by exploring complexes with DNA or liposomal encapsulation. These methods have not yet yielded results practical for adoption into clinical practice but are being actively studied. Such efforts are based on the hypothesis of Trouet et al (16) that one can enhance the selectivity of these anticancer drugs by taking advantage of enhanced absorption of DNA-drug complexes (subsequently cleared) into tumor cells contrasted with that of normal cells. Liposomes have the added theoretical advantages that changes in surface charge may selectively inhibit absorption into cardiac tissues, for example, as opposed to tumor (17).

Finally, one needs to consider the possibility of improving the therapeutic index of DNR by the use of agents that might protect against cardiotoxicity, particularly if it is related to free-radical damage. Such agents might include vitamin E, N-acetylcysteine, or coenzyme Q 10. Although such studies are ongoing with doxorubicin, it is premature to state whether or not such protection may be achieved clinically.

CONCLUSIONS

Future investigations with DNR must be viewed in the context of other anthracyclines and related drugs. Several have been introduced clinically (rubidazone, doxorubicin, AD-32, carminomycin, aclacinomycin A, 4′-epi-doxorubicin, and detorubicin). In addition, related intercalating drugs are also under study (m-AMSA and dihydroxyanthracenedione). These drugs share with the anthracyclines biochemical effects of inhibiting thymidine and uridine incorporation at very low concentrations as well as the ability to bind to DNA as documented by change in thermal melting temperature (Tm) of calf thymus DNA. Their preclinical spectra of activity also share similarities: cross-resistance has also been demonstrated. On the other hand, clinical experience shows important toxicologic differences for these synthetic compounds (no cardiomyopathy to date, and no tissue necrosis upon extravasation), and in the case of m-AMSA, the cross-resistance with anthracyclines has not been absolute.

Notwithstanding new drug development, new roles for DNR might be considered from laboratory or from clinical investigations in the following areas:

Laboratory Investigations

A number of comparative parameters other than antitumor efficacy in murine tumors are being investigated. Studies of DNA binding, cellular uptake, cell cycle specificity, and pattern of inhibition of macromolecular synthesis, as well as differential effects on the immune system may indicate important differences among the various drugs that may alter their selectivity for normal and malignant tissues. The alkaline elution technique introduced by Kohn and coworkers (18) has also been helpful in probing the comparative effects of various anthracyclines on tumor cell DNA (19).

Phase II Clinical Studies

Comparative studies with single agents frequently have low priority for clinical investigation. However, comparisons of DNR with rubidazone alone or in combination, and with doxorubicin, in leukemia are ongoing. These compounds are quite similar, however, and would be expected to yield quite similar results. Greater activity for some anthracyclines has been attributed based on clinical experience in specific conditions. In the absence of comparative data, these cannot be regarded as firm. Nonetheless, doxorubicin has unquestionably demonstrated greater activity against solid tumors, and there is little justification to pursue studies with DNR in this area at present.

Clinical Pharmacology

Delineation of human pharmacology and metabolism may bring out additional directions for future study. AD-32's metabolism bears a similarity to other anthracyclines with its conversion to an alcohol following the action of aldo keto reductase (13). Variations in pharmacokinetics among individual patients also need to be explored since this may have implications in predicting both tolerance and response.

Anthracyclines have become firmly established in clinical practice for the treatment of certain diseases. In particular, DNR is an important addition to the treatment of the various types of acute leukemia. Further progress may be expected with the introduction of new anthracyclines and careful analysis of clinical experience in relation to preclinical studies.

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A Review of the Preclinical Antitumor Activity and Toxicology of L-Asparaginase Derived From *E. coli*

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SUMMARY

The development of L-asparaginase as an antineoplastic agent is reviewed herein. Included is a brief summary of the early work performed by Kidd and Broome. The major emphasis is on describing the activity that L-asparaginase derived from *Escherichia coli* has shown against a number of preclinical tumor models and the comparative toxic effects it has produced in mice, rats, rabbits, dogs, and monkeys.

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The discovery of L-asparaginase represented a major advance in the treatment of cancer for two principal reasons: first, this agent demonstrated reproducible activity against a number of animal tumor systems and, second, it appeared to exert its antitumor effect selectively, sparing normal tissues.

L-Asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. Although L-asparagine is not considered to be an essential amino acid for normal mammalian tissue systems, studies dealing with the mechanism of action of L-asparaginase have identified several tumor cell lines that possess low asparagine synthetase activity (1). These abnormal cell lines have been shown by a number of investigators to be dependent on external sources of L-asparagine for viability and are, therefore, susceptible to the depletion of this amino acid produced by the action of L-asparaginase (2–7).

The earliest work in this area was performed by Kidd (8) in 1953 and involved the use of whole guinea pig serum in C3H mice inoculated with Gardner lymphosarcoma (6C3HED lymphoma) (fig 1). In 1961, Broome (2) proved that the antitumor principle present in guinea pig serum was L-asparaginase (9). Large scale research efforts were somewhat hampered until 1963 when Mashburn and Wriston (10) identified a more practical source for the enzyme by showing that the L-asparaginase derived from *Escherichia coli* possessed activity, equivalent to that of guinea pig serum, against the 6C3HED lymphoma cell line (fig 2). It was later determined that the antitumor activity resided with the enzyme fraction showing a high substrate affinity, designated EC-2, with the low substrate affinity fraction, EC-1, being inactive (4) (table 1). L-Asparaginase has been isolated from many sources other than the two listed above (table 2). While the enzyme preparations derived from yeast and *B. coagulans* have failed to demonstrate any antitumor effect in the systems tested, significant activity was achieved using the preparation obtained from *S. marcescens* and using a preparation obtained from *Erwinia carotovora*. Although four active species exist, the bulk of all the preclinical and clinical work has been done with the enzyme derived from *E. coli*.

ANTITUMOR ACTIVITY

L-Asparaginase has been shown to be active against more than 50 mouse tumors, three rat tumors, and the canine lymphosarcoma. A partial listing of the systems showing sensitivity has been abstracted from the literature and appears in table 3. Although the majority of these susceptible tumors are derived from the lymphoid cell line of the mouse, the rat sarcoma ACMC-42 and Walker 256 carcinosarcoma both show sensitivity to the enzyme. On the other hand, a larger number of morphologically similar murine tumors proved resistant to the effects of L-asparaginase. A partial listing of those resistant tumor lines is provided in table 4. This data subset has been adapted from the extensive summary developed by Adamson and Fabro (1). Their review of the response data for 109 mouse leukemias in-

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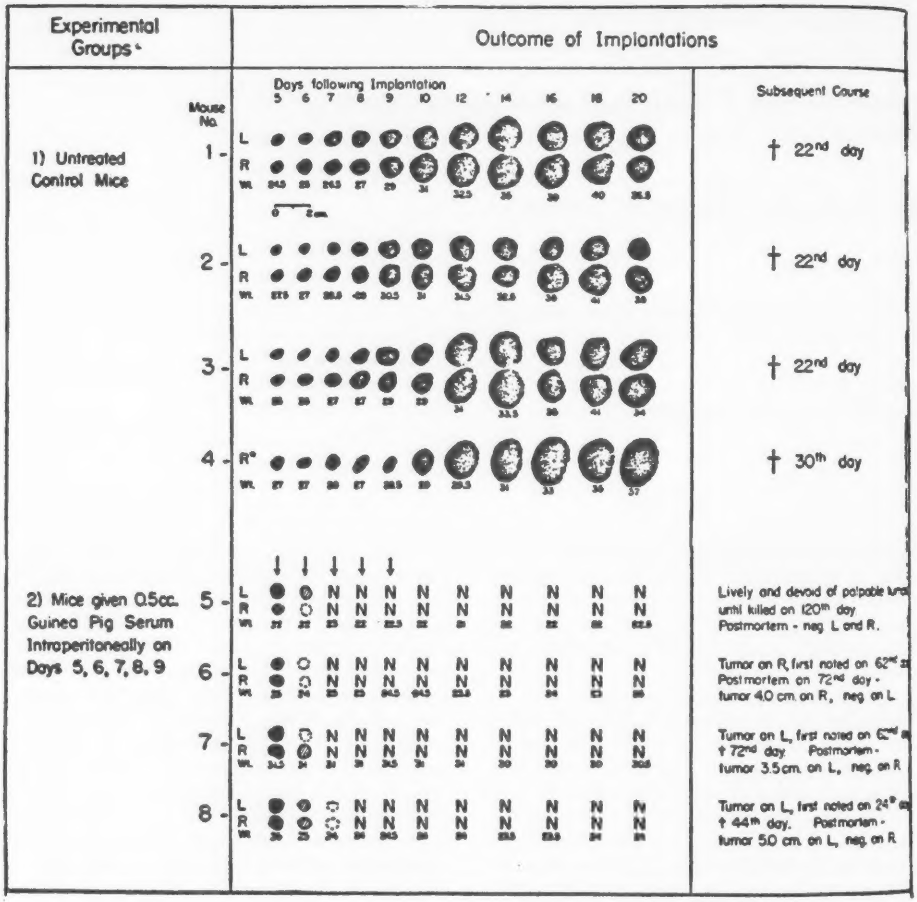
Experimental Groups*
Outcome of Implantations
Days following Implantation
5 6 7 8 9 10 12 14 16 18 20
Mouse No.
Subsequent Course
1) Untreated Control Mice
1 -
L
R
Wt.
0 2cm.
† 22nd day
2 -
† 22nd day
3 -
† 22nd day
4 -
R*
† 30th day
2) Mice given 0.5cc. Guinea Pig Serum Intraperitoneally on Days 5, 6, 7, 8, 9
5 -
N
Lively and devoid of palpable tu
until killed on 120th day
Postmortem - neg. L and R.
6 -
Tumor on R, first noted on 62nd
Postmortem on 72nd day -
tumor 4.0 cm. on R, neg. on L
7 -
Tumor on L, first noted on 62nd
† 72nd day. Postmortem -
tumor 3.5 cm. on L, neg. on R
8 -
Tumor on L, first noted on 24th
† 44th day. Postmortem -
tumor 5.0 cm. on L, neg. on R

FIGURE 1.—Regression of subcutaneous lymphosarcoma (6C3HED) in mice given guinea pig serum intraperitoneally (Kidd, 1953).

TABLE 1.—Effect of asparaginases from agouti serum and *E. coli* on the growth of lymphoma 6C3HED*

Treatment	Number of tumors implanted	Number of tumors completely suppressed	Days of tumor appearance	Standard deviation (days)
No enzyme	26	0	9	±1
Agouti serum (0.2 ml) 150 units	6	0	13	±1
E. coli high- + low-affinity enzymes (0.25 ml) 96 units	8	3	16	±2
E. coli low-affinity enzyme (0.45 ml × 2) 168 units	6	0	9	±0

*Asparaginase activity was assayed by ammonia production at a concentration of asparagine of 10 mM. Units of enzyme: moles of ammonia formed per hour. Suspensions containing 50,000 Lymphoma 6C3HED cells were injected subcutaneously into C3H mice on Day 0. Enzyme was given intraperitoneally on Day 1 or Days 1 and 2 to provide the total number of units shown (Schwartz et al, Biochemistry 56:1518, 1966).

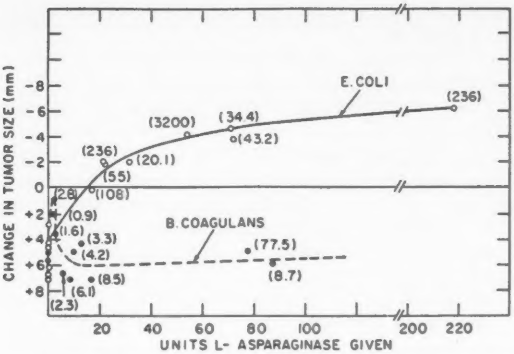

FIGURE 2.—Effect of L-asparaginase on growth of tumor (Mashburn and Wriston, 1963). Numbers in parentheses refer to specific activity (unit L-asparaginase/mg protein) of samples tested. All points are the average of 4 animals.

TABLE 2.—Sources of L-asparaginase

Source of L-asparaginase	Plasma half-time after iv administration (hrs)	Anti-tumor activity
Guinea pig serum	19–26	+
S. marcescens		+
E. coli EC-2	2.5	+
E. coli EC-1	*	–
Yeast	0.5	–
B. coagulans		–

*The plasma half-time of EC-1 L-asparaginase could not be determined in the mouse after intraperitoneal injection, either-because it was cleared rapidly or not absorbed (Adamson and Fabro, 1968).

TABLE 3.—Types of experimental tumors sensitive to L-asparaginase

Tumor	
Mouse leukemia	P1534
	L5178 Y
	ERLD
	EARAD 1
	ASL 11
Mouse lymphoma	Lorenz's lymphoma
Mouse lymphosarcoma	6C3HED
	P1798
Rat lymphosarcoma	Murphy-Sturm
Dog lymphosarcoma	Spontaneous
Rat sarcoma	ACMC 42
Rat carcinosarcoma	Walker 256

TABLE 4.—Mouse tumors insensitive to L-asparaginase

Tumor	
Leukemia	L1210
	RADA1
	K36
	ELS1
	ELS4
	SJL G1
	EL 4
Sarcoma	S 180
	Meth A
Plasma cell	OPC
	YPC-1

dicated that cell sensitivity was related in some way to the mode of tumor induction. As can be seen in table 5, 5/19 of those tumors arising spontaneously and 21/48 of those tumors induced by x-irradiation were sensitive to the enzyme, while none of 16 tumors induced by four viruses and only two of 24 tumors induced by the two chemical agents shown were sensitive to the effects of L-asparaginase. One interpretation of the data shown here would be that tumors induced by x-irradiation or arising spontaneously are more likely to be dependent on exogenous sources of L-asparagine.

Over the past three years, the National Cancer Institute has tested a number of the older chemotherapeutic agents, such as L-asparaginase, for activity in the current preclinical screening panel. As can be seen in table 6, data are lacking for the IV Lewis lung and human breast, colon and lung zenograph systems. Activity was demonstrated against the Walker 256 carcinosarcoma, but that model has been dropped from the panel because it was found to be too sensitive and it did not predict well for antitumor activity in humans. L-Asparaginase did show some activity against the P388 leukemia but was inactive against the other systems presently in use. Work done on cell cultures by Broome (2,3) and Boyse et al (4) showed that tumor cells that are sensitive to L-asparaginase require L-asparagine for optimal growth, while those tumor

TABLE 5.—Antitumor activity of guinea pig serum on 109 mouse leukemias

Mode of induction	Number of tumors found	
	Sensitive	Insensitive
Spontaneous	5	14
X-irradiation	21	27
Urethan	2	12
DMBA*	0	12
Virus†	0	16

*DMBA = 9,10-dimethyl-1,2-benzanthracene.
†Moloney, Gross, Friend, and Rauscher viruses.

Table 6.—NCI preclinical tumor panel

Tumor model			Activity	
P388	Leukemia	(IP)	Marginal	(136% T/C)
B16	Melanoma	(IP)	None	
CD8F1	Mammary	(SC)	None	
C38	Colon	(SC)	None	
L1210	Leukemia	(IP)	None	
Walker 256	Rat carcinosarcoma	(IP)	Yes	(287% T/C)
Lewis lung		(IV)	—	
Human tumor zenographs			—	

cells that grow in media lacking the amino acid are resistant to the action of L-asparaginase. Horowitz et al and Broome and Schwartz later demonstrated that L-asparaginase-sensitive tumors have little or no capacity to synthesize L-asparagine, while cells from resistant tumors can form L-asparagine using L-aspartic acid and ammonia or L-glutamine, with ATP serving as an energy source (5,7). Moreover, data compiled from several sources and reported by Adamson and Fabro (1) show that a sensitive tumor could be made resistant by exposure to subeffective doses of L-asparaginase, with cells from the now resistant tumor possessing L-asparagine synthetase activity (table 7). It has not been possible, however, to repress L-asparagine synthetase activity and induce sensitivity by exposing resistant cells to excessive levels of L-asparagine for several generations (11).

Table 7.—Relation between L-asparagine synthetase activity and L-asparagine requirement in vitro of murine tumor cells sensitive and nonsensitive to L-asparaginase*

Tumor	L-Asparagine synthetase activity (nmoles/mg protein/hr)	Requires exogenous L-asparagine in vitro	Inhibition of growth by L-asparaginase in vivo
Asparaginase sensitive			
L5178Y	0	+†	+
6C3HED	0	+	+
EARAD1	0.9	+	+
P1798	0	+	+
Asparaginase nonsensitive			
L1210	—	—	—
RADA1	156	-	-
BALD RL 2	39	—	—
K36	23	-	-
EL4	55	—	—
Meth A	65	—	—
Asparaginase resistant			
6C3HED-Res	34	—	—
EARAD1-Res	6.4	—	—
P1798-Res	5.8	—	—

*From Adamson and Fabro (1). Data from references 2, 31, 35, 36, 43; Adamson RH, and Fabro S, unpublished observations, 1967; Old LJ, personal communication, 1968.

†+ = yes; — = no.

Work performed by Adamson and Fabro also demonstrated a dose-response relationship with L-asparaginase. As one can see in Table 8, a single-treatment dose, administered at levels above 1000 IU/kg, produced apparent cures in all animals inoculated with L51784 leukemia, whereas smaller doses administered daily had no effect. It has also been shown by Dowley et al that, when given in sufficiently large doses, this agent will inhibit the growth of sensitive tumors implanted subcutaneously, intraperitoneally, or intracerebrally (12). Since L-asparaginase has a molecular weight of 139,000 and does not cross the blood-brain barrier to any significant extent, it must be presumed that the inhibitory effect exhibited against intracerebral neoplasms is due first to a depletion of blood L-asparagine and then indirectly to the cerebrospinal fluid L-asparagine levels.

Table 8.—Effect of EC-2 L-asparaginase treatment on the survival of mice bearing L5178Y leukemia*

Dose (IU/kg)	No. of 90-day survivors
Controls	0/10
12.5	0/10
25	0/10
50	4/10
100	8/10
1000	10/10
5000	10/10

*Mice were inoculated with 10^5 cells intraperitoneally; treatment started 24 hrs later and was continued daily × 5, except in the animals which received 1000 or 5000 IU/kg, who were treated on Day 1 only. Median survival time of controls was 17.5 days.

DRUG COMBINATION STUDIES

Table 9 characterizes the responses produced when L-asparaginase was combined with a number of other chemotherapeutic agents. As indicated, when given along with BCNU to mice inoculated intracerebrally with L5178Y or EARADI leukemia, the effect produced was better than that which would have resulted using either agent alone. An improved antitumor effect was also seen against the L5178Y ascites tumor when the combination included azaserine or azotyomycin, two glutamime antagonists; and a significant increase in survival was produced in mice bearing the P1798 lymphosarcoma when L-asparaginase was given prior to azaserine or vinblastine. No positive effect was seen, however, when these same agents were given simultaneously. Note that no beneficial effect was seen when mice bearing L1210, a naturally resistant tumor, were given DON, another glutamine antagonist, and L-asparaginase in combination. Note also that a negative therapeutic effect resulted when L-asparaginase was combined with methotrexate, 5-fluorouracil, or daunomycin.

TOXIC EFFECTS

As indicated previously, much of the initial enthusiasm for L-asparaginase arose from the belief that it was the first anticancer agent to exploit a qualitative difference between normal tissues and certain neoplastic cells. An impressive volume of toxicologic data has been compiled using several species, including the mouse, rat, guinea pig, rabbit, dog, and monkey. What follows is an attempt to summarize the salient features as they have presented themselves in each instance. Note that all species tested exhibited varying degrees of decreased activity, weakness, anorexia, and weight loss, in addition to the findings outlined below.

Mice

Mice were found to tolerate extremely large doses of L-asparaginase without any apparent damage to normal tissues. Acute toxic effects consisting of limb spread, flaccidity, and hyperpenia, were seen by Schein et al (13) only after intravenous doses in excess of 18,500 IU/kg were given. The LD50 established by Lorke and Tettenborn (14) was in excess of 200,000 IU/kg.

The major consistently observed adverse effect resulted from the ability of L-asparaginase to elicit an immune response, even at low doses, when given by repeated injections (15).

Rats

Rats were also found to tolerate large parental doses of the enzyme without developing significant toxic effects. As in the mouse, the LD50 established was above 200,000 IU/kg. Studies conducted by Cooney and Rosenbluth (15) show that a daily dose as large as 5000 IU/kg given × 5 produced no ill effects. At that dose, not even weight loss occurred. It is also noteworthy that, unlike the mouse, rats receiving L-aspa-

Table 9.—Combination studies

Agents	Tumor	Site	Effect	Reference	
L-Asparaginase + BCNU	L5178Y EARAD1 leukemia	IC	(+)	Burchenal & Dollinger	23
L-Asparaginase + azaserine	L5178Y	IP	(+)	Jacobs et al	24
L-Asparaginase + azotomycin			(+)		
L-Asparaginase + azaserine	P1798 lymphosarcoma	SC	(+)	Mashburn	25
L-Asparaginase + vinblastine					
L-Asparaginase + DON	L1210 leukemia	SC	(0)	Jacobs et al	24
L-Asparaginase + azaserine					
L-Asparaginase + methotrexate	P1798 lymphosarcoma	SC	(−)	Mashburn	25
L-Asparaginase + 5-FU			(−)		
L-Asparaginase + daunomycin			(−)		
L-Asparaginase + azaserine			(−)		

raginase daily for 15-90 days did not show signs of immunogenic toxicity (16). We do not intend to explore in any detail a probable explanation for this phenomenon except to say that many investigators have clearly documented the immunosuppressive properties of L-asparaginase in mice by showing that it can produce: 1) lymphopenia and atrophy of lymphoid tissues including the thymus and spleen, 2) a delay in the development of graft vs host reactions, 3) inhibition of the blastogenic response of lymphocytes to phytohemagglutinin, 4) delays in skin graft rejection, and 5) blunting of the formation of antibodies to sheep erythrocytes (17). Weksler and Weksler (18) have related these immunosuppressive properties to depletion of asparagine level. Cooney and Rosenbluth have postulated that, in the rat, the immunosuppressive properties of L-asparaginase overshadow its immunogenic potential. The enzyme was also found to be embryotoxic to this species by Adamson (19) and Lorke and Tettenborn (14).

Rabbits

The rabbit was the first species to contradict the notion that L-asparaginase had virtually no toxic effects on normal tissues. Oettgen et al (20) observed a syndrome consisting of pupillary constriction, profuse salivation, convulsions, respiratory paralysis, and death, occurring over a period of 24-48 hours after drug administration. Subsequent work by Tettenborn et al (21) and Young et al (22) identified the parathyroid as the organ targeted by the enzyme. The latter group showed that 1000 IU/kg, given daily, produced hypocalcemia in all of the animals tested, with 70% developing tetany. Most animals also exhibited hyperphosphatemia, hypomagnesemia, hypokalemia, and azotemia. Histologic and ultrastructural lesions of the parathyroid gland, consisting of degranulation and degeneration of chief cells and a deficiency of subcellular organelles associated with protein synthesis, were also documented by Young. Cooney and Rosenbluth (15) examined in vitro the effect of L-asparaginase on the rabbit parathyroid and found that it inhibited the synthesis of the enzyme L-asparaginase synthetase, which is normally found in the gland in high concentrations, and thereby effectively lowered local L-asparagine levels. It must be pointed out that all of the animals whose parathyroids were so affected did not develop hypocalcemia and tetany. The converse was true, however; all of the animals that exhibited the hypocalcemic syndrome showed low glandular L-asparagine levels.

Dogs

The toxicologic findings produced in beagle dogs paralleled those observed in mice and rats. Dogs were shown to be somewhat more sensitive, however, with an LD50 of 50,000 IU/kg. Doses up to 10,000 IU/kg were tolerated without significant adverse effect other than emesis. Adamson and Fabro safely gave 1000-2000 IU/kg daily × 14, but, as in the mouse, L-asparaginase readily induced immunogenic toxicity, and, as in the rat, it was found to be teratogenic (26).

Monkeys

The rhesus monkey was the first species to give an indication that L-asparaginase derived from *E. coli* was hepatotoxic. The principal morphologic abnormality, fatty infiltration, was consistently produced at a dose of 6.12 IU/kg administered daily for 5 days. All animals who eventually developed steatosis had previously shown abnormal retention of bromosulfophthalum. Serum glutamate oxalacetate transaminase and alkaline phosphatase levels were not useful in predicting the development of steatosis. Cooney and Rosenbluth have reported that the enzyme from *E. coli* reduces the level of total hepatic protein. They postulate that a failure of the liver to adequately synthesize sufficient lipoproteins, required to export lipid, contributes to the development of fatty infiltration (15).

Table 10 lists a number of dietary supplements that have failed to prevent the development of this enzyme-induced steatosis. We see included here: L-asparaginase, L-glutamine, choline ± α-tocopherol, Nutramigen plus Enfamil, and phenoxybenzamine.

The other toxic effects that have been consistently produced in the monkey are anemia and immunogenic stimulation. It is of interest to note that one monkey died of hemorrhagic colitis. Table 11 summarizes the major toxic effects observed in the five animals just discussed plus the data available for the guinea pig.

CONCLUSION

L-Asparaginase is an agent that has shown significant activity against a number of tumor systems primarily of lymphoid origin. The antitumor effect shown has been related to a lack of L-asparagine synthetase activity within the sensitive cell. This promotes a relative dependence on exogenous sources of L-asparagine.

The notion that this agent was nontoxic to normal tissues stemmed from the remarkable tolerance to the enzyme shown by mice, rats, and, to some extent, dogs. Subsequent work performed in other species, however, proved that L-asparaginase from *E. coli* produces definite toxic effects that are related to its ability to deplete L-asparagine levels and that the types of toxic effects seen vary with the species being observed.

Table 10.—Failure of dietary supplements to prevent fatty livers in rhesus monkeys treated intravenously with EC-2 L-asparaginase*

Supplement schedule†	Dose of L-asparaginase (IU/kg × days)	No. of animals	Incidence of	
			BSP‡ retention	Fatty liver
Pretreated with L-asparagine: 500 mg/kg/day × 3, p.o., followed by daily administration of 500 mg/kg × 5, p.o., 2 hr pre L-asparaginase	1000 × 5	1	1	1
Pretreated with L-asparagine: 500 mg/kg/day × 7, p.o., followed by daily administration of 500 mg/kg × 5, p.o., 2-3 hr pre L-asparaginase	1000 × 5	1	1	1
Pretreated with L-glutamine: 500 mg/kg/day × 3, p.o., followed by daily administration of 500 mg/kg × 5, p.o., 3-5 hr pre L-asparaginase	1000 × 5	1	1	1
Pretreated with L-glutamine: 200 mg/kg/day × 4, p.o. and 150 mg/kg/day × 4, s.c., B.I.I. (2-hr interval) followed by daily treatment with L-glutamine 200 mg/kg/day, p.o. 2 hr before L-asparaginase and 100 mg/kg s.c., t.i.d. 1 hr before and 1 and 2-3 hr post L-asparaginase	1000 × 5	1	1	1
Pretreatment with choline chloride: 100 mg/day × 4, p.o., followed by daily administration of 100 mg/kg, p.o., 2-3 hr pre L-asparaginase	1000 × 5	1	1	1
Pretreatment p.o. for 7 days with 200 mg/kg b.i.d. choline chloride and 20 units/kg, b.i.d., α-tocopherol acetate followed by same p.o. doses given daily 2 hr previous and 5 hr post L-asparaginase	1000 × 5	1	1	1
Pretreated with α-tocopherol acetate, p.o.: 20 units/kg/day × 7 followed by daily administration of 20 units/kg/day, p.o., 2-3 hr before L-asparaginase	1000 × 5	1	1	1
Forced feeding of Nutramigen and Enfamil equivalent to 300 cal/day (150 cal before and 5-6 hr post L-asparaginase) × 5	1000 × 5	1	1	1
Phenoxybenzamine p.o., 20 mg/kg b.i.d. × 52 hr pre and 5 hr post L-asparaginase	1000 × 5	1	1	1

*From Cooney and Handschumacher, 1970.

†p.o.—orally; s.c.—subcutaneously; t.i.d.—3 times a day; b.i.d.—twice a day.

‡BSP—bromsulfophthalein.

Table 11.—Summary of organ toxicity

Species	CNS	Anemia	Leukopenia	↑BSP↑	SGOT	Fatty liver	Immune response	Reference	
Rhesus monkey	−	++	±	++	±	++	+	Schein et al	13
Beagle dog	−	+	−	−	−	−	++	Schein et al	13
Rabbits*	+++	−	−	−	−	−	−	Oettgen et al	20
								Tettenborn et al	21
								Young et al	22
								Cooney & Rosenbluth	15
								Adamson & Fabro*	26
Guinea pigs	−	−	−	−	−	−	++	Schein et al	13
Mice	−	−	−	−	−	−	++	Schein et al	13
Rats*	−	−	−	−	−	−	−	Schein et al	13
								Adamson*	19

*Embryotoxicity has been shown.

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Pharmacology of L-Asparaginase and the Effects of Host and Enzyme Modification[1]

Jack R. Uren[2,3,*]

SUMMARY

Pharmacology is the study of the properties of a drug with respect to its interaction with the host, toxicology, and with the disease, therapeutics. Since both the toxicology and therapeutic activity of L-asparaginase are presented by others at this symposium, I will give only a brief overview of the pharmacology of L-asparaginase with particular reference to how it differs from other chemotherapeutic agents. Primarily, I would like to discuss how it may be possible to modify some of the pharmacological constraints on the therapeutic responses to L-asparaginase by combining the enzyme with inhibitors of asparagine synthetase and by using polymer-modified forms of the enzyme.

[Cancer Treat Rep 65 (Suppl 4):47–55, 1981]

L-Asparaginase is unique among cancer chemotherapeutic agents for at least two reasons. First, the enzyme can exert its cytotoxic activity at sites far removed from the tumor itself. The evidence for this conclusion is based on the fact that the cytotoxicity observed by transplanting an asparaginase-sensitive cell in asparagine deprived media is equivalent to that obtained by adding asparaginase to growth media (1). This is in contrast to all other clinically used chemotherapeutic agents which either penetrate or act on the surface of the affected cells. Secondly, L-asparaginase is a catalytic protein. Under physiological conditions, one molecule of *Escherichia coli* L-asparaginase will degrade 90,000 molecules of L-asparagine per minute (2). These catalytic rates are a consequence of a complementary surface on the enzyme that binds and distorts reactant molecules. Surface catalysis also produces a high degree of substrate specificity. For example, D-asparagine and L-glutamine are hydrolyzed at only 4% the rate of L-asparagine, and L-aspartic acid is not inhibitory to the *E. coli* enzyme (3). These characteristics are in marked contrast to those of other chemotherapeutic agents that need to be administered in many thousandfold excess over their target molecules and show only limited selectivities.

Surface catalysis also requires that the enzymes providing underlying structural support be large molecules. The macromolecular nature of enzymes greatly affects their pharmacologic properties. In vivo, L-asparaginase distributes almost entirely in the plasma volume. Consequently, host tissues which can synthesize asparagine and feed neighboring malignant cells can function as sanctuaries for these tumors. Figure 1 illustrates this difficulty. John Fulkerson, working with Robert Handschumacher at Yale University, determined by bioassay the number of surviving tumor cells in various organs of mice three days post a 3-day treatment regimen with either L-asparaginase (200 U/kg) or saline. Over this time period, L-asparaginase was effective in eliminating the tumor from all organs examined in the host except the liver. The liver has also been shown to be the organ for plasma asparagine synthesis in the host (4).

The macromolecular nature of enzymes is also a prerequisite property for immunogenicity. Whereas all drug molecules can be antigenic, only macromolecular antigens are immunogenic. This immunogenicity can express itself in two ways. If IgG antibodies are

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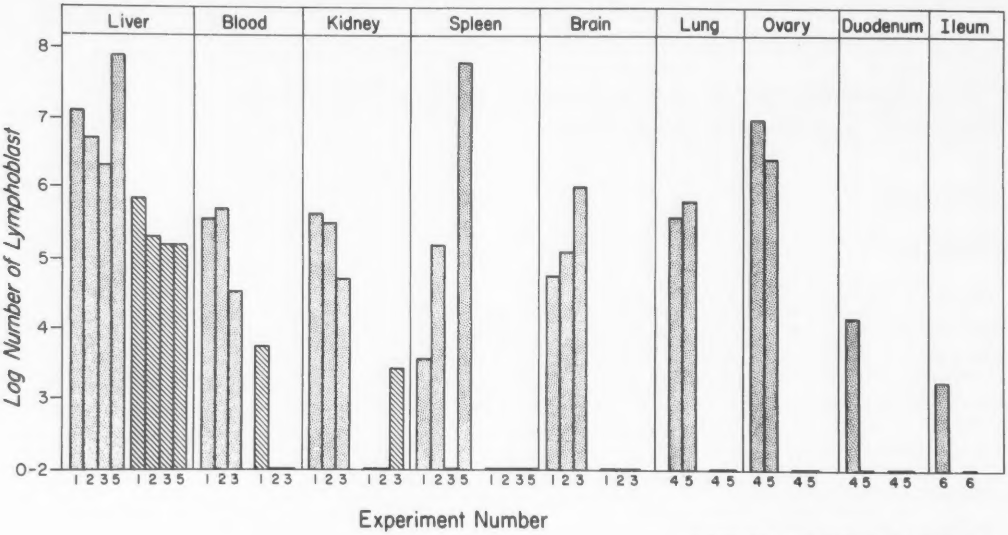


* Data from John Fulkerson, M.D. Thesis, Yale University, 1972

FIGURE 1.—Tissue distribution of L5178Y lymphoblasts in asparaginase-treated and non-treated mice 3 days following therapy. On Day 0 10^3 L5178Y lymphoblasts were injected via the tail vein into AKD$_2$F$_1$ female mice. On Days 8, 9, and 10 half of the mice were treated with 200 U/kg L-asparaginase iv and the other half were treated with saline. On Day 13 the mice were sacrificed; organs were homogenized with Fisher's media; and aliquots were injected ip into ADK$_2$F$_1$ mice. Survival times were compared to those when a known number (10^2 to 10^6) of L5178Y cells were injected ip into AKD$_2$F$_1$ mice. Data from John Fulkerson, MD, thesis, Yale University, 1972.

produced, rapid clearance of the enzyme-antibody complex is observed and a pharmacologically active dose of the enzyme cannot be administered (5,6). Whereas, if IgE antibodies are produced, symptoms of anaphylaxis are exhibited such that therapy must be discontinued (5,6). Both IgG and IgE reactions have been observed in patients, with neither being predictive of the other (7). Since the proper functioning of the immune system requires asparagine, the enzyme is also immunosuppressive (8). Consequently, immunologic toxic effects are observed only in approximately 25% of the patients. Nevertheless, the hypersensitive reactions are, in fact, the most undesirable side effect in asparaginase therapy (6). The other host toxic manifestations can generally be related to the inhibition of the synthesis of several important polypeptides and proteins. These toxic reactions include hypoglycemia (9), defective bloodclotting (10), hypoalbuminemia (11), and hypocalcemia (12). Unlike most other chemotherapeutic agents, however, L-asparaginase causes little cytotoxicity to bone marrow stem cells, oral and gut mucosa, and hair follicles, making it ideal for combination therapies (10).

Resistance to therapy also occurs via genetic mechanisms. In asparaginase therapy, asparagine-independent sublines which occur with a frequency of one cell in 10^6 in a nonmutagenized population (1) are selected. These asparaginase-resistant cells express their asparagine synthetase levels as a regulated response to the level of asparagine in the growth media, much like most normal tissues (13).

COMBINED THERAPY OF L-ASPARAGINASE WITH ASPARAGINE SYNTHETASE INHIBITORS

Since the constraints on asparaginase therapy by both genetic resistances and host liver sanctuaries have as their basis the level of asparagine synthetase activity in the tumor or the host tissue, inhibitors of asparagine synthetase may increase the effectiveness of such therapy and expand the spectrum of tumors that respond to it. Because a priori inhibitors of asparagine synthetase destroy the original difference between normal and malignant tissues, enhanced toxic effects can be expected from such therapy. Nevertheless, synergistic antitumor responses might be observed with proper dose scheduling or through inherent differential sensitivities.

Asparagine synthetase is regulated in vivo both at the genetic level with repressional control by asparagine, or its metabolite acting as a co-repressor, and at the enzyme level with product inhibition by asparagine (13,14). Consequently, asparagine analogs might be good candidates to either inhibit the enzyme or prevent its derepression, or both.

Figure 2 illustrates the growth kinetics for an asparaginase-resistant leukemic cell line when deprived of asparagine. This figure shows a lag in the cell-growth kinetics that correlates with the time required for a twofold derepression in asparagine synthetase activity. Consequently, compounds that selectively inhibit asparagine synthetase or its derepression should show a corresponding inhibition of cell growth. In the presence of asparagine in the growth media, however, cell growth should be independent of asparagine synthetase activity, and any observed cell-growth inhibition should be reversed.

Using this reversible cell-growth inhibition assay, nineteen possible asparagine analogs were screened. Table 1 (15) summarizes these results. The compounds that demonstrated reversible cell-growth inhibition were (in decreasing order of effectiveness): L-β-aspartyl methylamide > D,L-β-aspartyl hydroxamate > D,L-β-aspartyl hydrazide > D,L-threo-β-hydroxy asparagine > D,L-threo-β-methyl asparagine > D,L-α-N-methyl asparagine. Compounds such as 5-chloro-4-oxo-norvaline are strongly inhibitory, but the ability of asparagine to reverse this inhibition is not consistent with its ability to inhibit other glutamine-requiring enzymes (16). The ability of these reversible inhibitory compounds to inhibit asparagine synthetase activity in vitro is shown in table 2 (15). The order of effectiveness observed in vitro was the same as that observed in cell culture except for D,L-threo-β-hydroxy asparagine. This compound was shown to be able to inhibit the charging of asparaginyl-tRNA but not aspartyl-tRNA. These compounds would also prevent the derepression of asparagine synthetase, but it was not possible to discern whether they were functioning as fraudulent co-repressors or through generalized protein synthesis inhibition brought about by intracellular asparagine depletion.

The most potent of these inhibitors was also quite resistant to L-asparaginase hydrolysis as shown in table 3 (15). Although β-aspartyl methylamide was the best inhibitor of asparagine synthetase (Ki = 200 μM) and most resistant to asparaginase hydrolysis, it was also an excellent inhibitor of L-asparaginase (Ki = 30

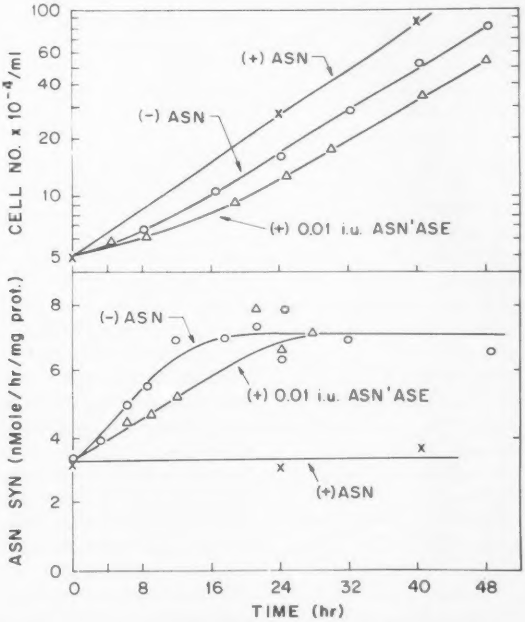
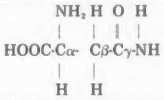


FIGURE 2.—Effects of asparagine removal on the growth of asparagine-independent cells and asparagine synthetase-specific activity. From Uren, Summers, and Handschumacher.

TABLE 1.—Structure activity comparison for various asparagine analogs towards the growth of an asparagine-independent L5178Y cell line in culture in the presence and absence of asparagine

Asparagine



$$HOOC\text{-}\underset{H}{\overset{NH_2}{C\alpha}}\text{-}\underset{H}{\overset{H}{C\beta}}\text{-}\overset{O}{\overset{\|}{C\gamma}}\text{-}\overset{H}{NH}$$

Analog structure	Growth inhibitory activity						
Substitution for H	Cα		Cβ		Cγ	Cγ	
	Dextro	Levo	Threo	Erythro		Cγ structure	Activity
$-CH_3$	NI*	WR†	WR	NI	SR‡	$-\overset{O}{\overset{\|}{C}}\text{-}N(CH_3)_2$	NI
$-OH$	—	—	SR	—	SR	$-\underset{CH_3}{\overset{O}{S}}=NH$	NI
$-NH_2$	—	—	NI	NI	SR	$-\overset{O}{\overset{\|}{C}}\text{-}CH_3$	SI
$-CH_2CH_3$	—	—	—	—	NI	$-\overset{O}{\overset{\|}{C}}\text{-}H$	SI
$-CH(CH_3)_2$	—	—	—	—	NI	$-\overset{O}{\overset{\|}{C}}\text{-}CH_2Cl$	SI
$-CH_2CH_2Cl$	—	—	—	—	SI§	$-\overset{O}{\overset{\|}{C}}\text{-}OCH_3$	NI

*NI = No growth inhibition.
†WR = Weak reversible inhibition.
‡SR = Strong reversible inhibition.
§SI = Strong irreversible inhibition.

TABLE 2.—Inhibition of asparagine synthetase in vitro

Addition	Activity (nmoles/hr)	% inhibition
None	4.43	—
L-Asparagine	0.38	91
L-β-Aspartyl methylamide	1.46	67
D,L-β-Aspartyl hydroxamate	2.72	39
D,L-β-Aspartyl hydrazide	3.81	14
D,L-β-Threo-β-methyl-asparagine	4.03	9
D,L-γ-N-methyl-asparagine	4.25	4
D,L-Threo-β-hydroxy-asparagine	4.50	0

μM). Consequently, therapy with this analog at levels that would inhibit asparagine synthetase would nullify any asparaginase therapy. This fact, together with its rapid in vivo metabolism by N-acetylation and excretion in the urine, limited its therapeutic usefulness. Nevertheless, as shown in table 4 (15), the analog by itself did show some therapeutic activity against asparaginase-resistant tumors. These results support the proposal that the antitumor activity of inhibitors of asparagine synthesis may be applicable to a spectrum of tumors broader than that which will respond to asparagine alone. Obviously, more potent inhibitors of asparagine synthetase that are neither hydrolyzed by nor inhibitory of L-asparaginase are necessary to reinforce this conclusion.

A new experimental lead in this direction is presented in figure 3. L-Homoserine-β-adenylate was synthesized by Dr. Chang at Yale University as an analog of the enzyme intermediate, aspartyl-β-adenylate, proposed by Meister (14). This compound

TABLE 3.—Analog hydrolysis by asparaginases

Substrate	E. coli		Erwinia carotovora	
	μmoles/min	%	μmoles/min	%
L-asparagine	10.1	100	79	100
D,L-β-aspartyl hydroxamate	4.1	40	18	23
D,L-β-aspartyl hydrazide	0.22	2.2	2.9	3.7
L-β-aspartyl methylamide	0.16	1.6	1.3	1.7

TABLE 4.—Antitumor activity of β-aspartyl methylamide*

Treatment		Saline		β-Aspartyl methylamide		
Tumor	Host	Weight change† (%)	Mean survival time (days)	Weight change† (%)	Mean survival time (days)	Percent ILS‡
L1210	BDF	+15	7.2 ± 0.4	0	10.6 ± 0.4	47
S180	CDI	+14	10.8 ± 1.1	+6	14.2 ± 0.8	31
AD755	AKD	+12	12.2 ± 1.0	+1	21.8 ± 1.0	79

*Groups of 5 mice were given intraperitoneal injections of 10^6 tumor cells and 0.1 ml of sterile saline or analog (300 mg/kg) solution twice daily on Days 2-5.

†Percent weight loss or gain (weight on Day 5/weight on Day 1 × 100).

‡Percent ILS (increase in life span) treated ÷ control × 100 − 100.

shows a thousandfold better inhibition constant than does β-aspartyl methyl amide for asparagine synthetase (17). Unfortunately, the compound does not show reversible cell growth inhibition in culture but does show evidence of metabolism to adenosine. A nonmetabolizable analog of this structure may possibly be the desired compound.

PHARMACOLOGY OF POLYMER-MODIFIED FORMS OF L-ASPARAGINASE

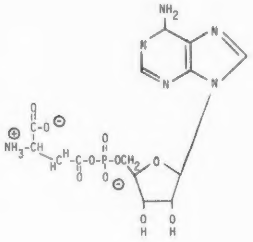
As indicated previously, immunologic toxic effects are the single most undesirable side effect from asparaginase therapy. These effects range in severity from mild allergic reactions to anaphylactic shock, often with accompanying rapid enzyme clearance. Consequently, asparaginase therapy is managed to minimize these complications. Patients are given short courses of enzyme for remission induction or consolidation as opposed to continued administration for maintenance therapy. In addition, patients are treated with combination chemotherapeutic agents, many of which are immunosuppressive. When allergic reactions do occur, therapy is switched to an enzyme isolated from another source that does not share cross-reacting antigenic determinants, usually from the *E. coli* enzyme to the *Erwinia carotovora* enzyme. Therefore, any procedure that greatly decreases the immunogenicity of the enzyme can not only remove the allergic reactions associated with its use but also broaden the ways in which, and expand the time over which, it can be effectively given. In this regard, we have examined the pharmacologic and immunologic consequences of attaching D,L-alanine polymers to the surface of both the *E. coli* and *Erwinia* L-asparaginases.

Figure 4 illustrates a proposed model to explain some of our enzymatic and biological results. We have attached a nonimmunogenic polypeptide (18) of D,L-alanine (M. wt. ~2000) to roughly 20 lysine residues by D,L-alanine-N-carboxyanhydride polymerization. These enzyme preparations have shown little change in Km with only a 50% loss in specific activity with a slightly more alkaline pH optimum. They are over a hundredfold more resistant to trypsin digestion and about sixfold more resistant to α-chymotrypsin digestion. The effects of the polymer attachment on plasma enzyme clearance and asparagine depletion is presented in figure 5. Both modified forms of the enzymes show about a sevenfold longer plasma half-life than the native enzymes. In addition, plasma asparagine depletion was extended 5 days longer for the modified *E. coli* enzyme and 10 days longer for the modified *Erwinia* enzyme over that of the native enzyme following the injection of 10 IU of each enzyme.

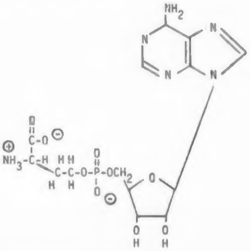
This greatly improved substrate depletion should predict an improved therapeutic response for the modified as opposed to the native enzyme at equivalent dosages. This result was not always observed. A possible reason was that tumor-bearing animals exhibited a much longer plasma half-life for the native en-

Asparagine Synthetase Reaction: $ASP + ATP + NH_3 \xrightarrow{Mg^{++}} ASN + AMP + PPi$

$ASP + ATP + GLN \xrightarrow{Mg^{++}} ASN + AMP + PPi + GLU$

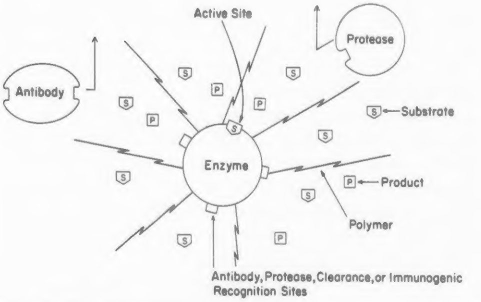


L-Aspartic acid-β-adenylate (substrate)
(JBC 247, 6708, 1972)



L-Homoserine-β-adenylate (Ki=0.2 μM)
(Cancer Treatment Reports, 63, 1095, 1979).

FIGURE 3.—Asparagine synthetase reaction requirements and structure of a proposed intermediate and inhibitor.


Active Site
Protease
Antibody
Substrate
Enzyme
Product
Polymer
S
P
Antibody, Protease, Clearance, or Immunogenic Recognition Sites

Possible Advantages Of Polymer Modified Enzymes

1. Mask Antigenic Determinants
2. Mask Immunogenic Recognition Sites
3. Mask Protease Susceptible Sites
4. Mask Clearance Recognition Signals
5. Allow Free Access To Small MWt. Substrates
6. Maintain Systemic Injectability
7. Alter pH Optimum By Changing Microenviroment

FIGURE 4.—Proposed model to explain the enzymatic and biological properties of poly-D,L-alanyl-asparaginases. From Uren and Ragin.

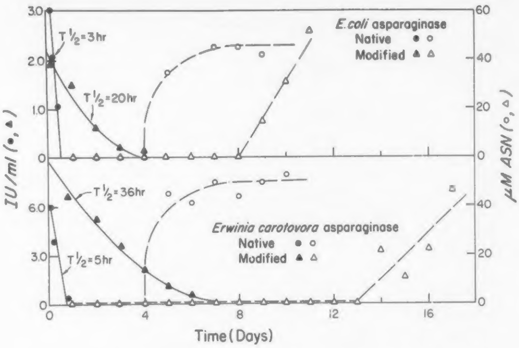


FIGURE 5.—Effect of poly-D,L-alanyl modification of *E. coli* and *Erwinia* L-asparaginases on plasma half-life and asparagine concentration in BDF_1 mice. From Uren and Ragin.

zyme (24 hrs as opposed to 3 hrs), probably due to the presence of the LDH-elevating virus in our tumor line (19). A parallel prolongation in the half-life of our modified enzyme was not observed. Consequently, only modified enzyme preparations that showed plasma half-lives in excess of 24 hrs demonstrated superior therapeutic activities; otherwise, activities similar to those of the native enzyme were observed. Table 5 (20) supports these conclusions. If therapy was initiated early, before the virus could exert its plasma half-life extending ability, both modified enzymes were superior to their native counterparts. If therapy was delayed until after the virus had established itself, only the modified *Erwinia* enzyme (t½ = 36 hrs) was superior to its native counterpart. The modified *E. coli* enzyme (t½ = 20 hrs) was equivalent to its native counterpart. When the more extensively modified *E. coli* enzyme (t½ = 36 hrs) was used for therapy, a greatly improved therapeutic activity was observed even with late therapy (table 6) (20). This preparation appeared to be about tenfold better than the native enzyme and was capable of curing mice with a single injection of 400 IU/kg.

The immunogenic properties of both the native and modified *E. coli* enzyme preparations were determined following inactivation with site-specific reagent 5-diazo-4-oxo-L-norvaline to remove the immunosuppressive activity of the enzymes. Table 7 (20) shows these results. Ouchterlony precipitin reactions were measured weekly after immunization with 20 μg of the antigens in complete Freund's adjuvant. These results show that it took over 4 weeks for precipitating antibodies to be elicited by the modified enzyme as opposed to 2 weeks for the native enzyme. Antisera derived from immunization with the modified enzyme displayed a reaction of identity between the modified and native proteins but gave no precipitin line with poly-D,L-alanine.

TABLE 5.—Percent increase in life span when mice bearing the asparaginase sensitive L5178Y leukemia are treated with various native and modified asparaginases

	Early treatment*		Late treatment†	
Enzyme	E. coli‡	Erwinia	E. coli‡	Erwinia
Native	22 ± 2	12 ± 2	45 ± 5	28 ± 4
Modified	39 ± 4	56 ± 4	42 ± 4	76 ± 6

*10^6-L5178Y cells implanted into each of 5 BDF_1 male mice on Day 0 and treated 1 hr post implantation with 10 IU of each enzyme.

†Treatment as above but 24 hrs post tumor implantation.

‡Modified *E. coli* asparaginase preparation 2 was used (plasma t½ = 20 hrs).

TABLE 6.—Effect of *E. coli* asparaginase modification on therapy of murine leukemia L5178Y*

	% Increase in life span	
Dosage IU/mouse	Native	Modified†
10	54 ± 9	>223‡
1	16 ± 3	50 ± 7
0.1	−2 ± .3	15 ± 2
0.01	2 ± .3	−5 ± .9

*10^6-L5178Y cells per mouse were implanted ip into BDF_1 male mice on Day 0 and treated ip bolus on Day 1. Each group contained 5 mice except for 10 control mice. Initial mouse weights averaged 25 g each.

†Modified *E. coli* asparaginase preparation 1 was used (plasma t½ = 36 hrs).

‡3 out of 5 60-day survivors.

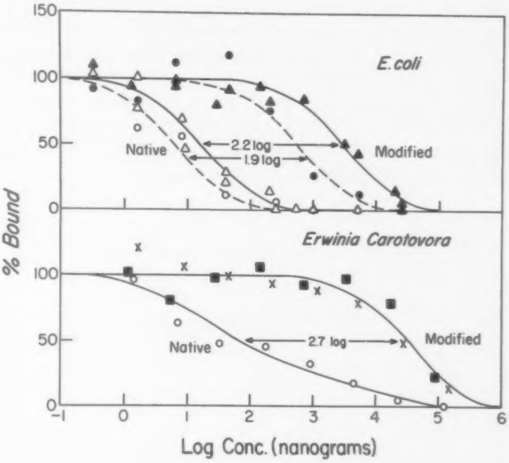


FIGURE 6.—Effects of polymer-modified asparaginases on competitive displacement of native enzyme from antisera derived from immunization with native (—) and modified (-) enzymes. From Uren and Ragin.

A quantitative measure of the degree of cross-reaction between the native and modified enzyme was determined by a radioimmunocompetition assay (fig 6). It took 300-fold more modified *E. coli* asparaginase than native enzyme to displace the radiolabeled native enzyme from antisera derived from animals immunized with the native enzyme and a hundredfold more modified enzyme when the antisera derived from mice immunized with the modified enzyme were utilized. Similarly, it took 500-fold more modified *Erwinia* enzyme than native to displace the radiolabeled native enzyme from antisera derived from mice immunized with the native *Erwinia* enzyme. If the modification procedure masked only some of the immunogenic determinants, we might expect immunization with the modified enzyme to elicit antibodies to only the unmasked determinants, and the native and modified proteins should be equivalent at displacing the radiolabeled antigen. This result is unlike the hundredfold difference observed. If all the antigenic determinants were masked on the modified enzyme and a 1% contamination by the native enzyme caused the displacement, we would not expect the antibody to be able to precipitate more than 1% of enzyme activity. In con-

TABLE 7.—Immunogenicity of native and modified (preparation 1 & 2) *E. coli* asparaginase in complete Freund's adjuvant with BDF_1 mice

Time	Native enzyme		Modified prep. 1		Modified prep. 2	
(wks)	Precipitin line	No. positive	Precipitin line	No. positive	Precipitin line	No. positive
1	None	0/25	None	0/19	None	0/5
2	Weak	25/25	None	0/19	None	0/5
3	Strong	25/25	None	0/19	None	0/5
4	Strong	25/25	Weak	15/19	None	0/5
5	Strong	25/25	Weak/strong	19/19	Weak	2/5

trast, 99% of the modified enzyme activity was precipitated by the antisera. Consequently, most of the antigenic determinants may still exist on the modified enzyme, but their ability to bind the antisera may be retarded by the steric effects of the polymer.

If the plasma clearance rates of the native and modified enzymes were re-examined in these immune mice, the results in figure 7 were observed. If early immune mice were used (ie, two immunization treatments), the native enzyme was immediately cleared from the plasma with no detectable circulating enzyme activity. With the modified enzyme, however, a prolonged plasma clearance was still observed. With highly immune animals (ie, four immunization treatments), both the native and the modified enzyme were immediately cleared with no detectable circulating enzyme activity. These results suggest that the modified enzyme would be useful in treatment of patients with low antibody titers against the native enzyme but not for patients with high antiasparaginase titers.

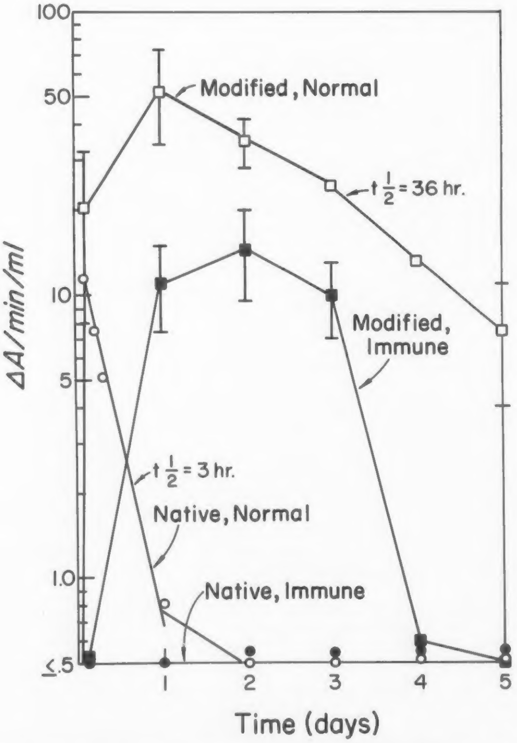


FIGURE 7.—Plasma clearance rate of native and modified *E. coli* asparaginase in normal mice and mice immunized with native enzyme. From Uren and Ragin.

Recently, we have examined the IgE and IgG responses of mice immunized with the native and modified *Erwinia* enzyme. Figure 8 shows these results. Passive cutaneous anaphylaxis (PCA) was used to measure antibody titers from the serum of immunized mice. Immunization with native inactivated enzyme at 10 mg/kg demonstrated an IgE titer of 80 at 2 weeks. This titer would gradually decay but could be stabilized by a secondary injection of 1 mg/kg at 4 weeks. In contrast, no observable IgE titers could be detected in the serum of mice immunized with the inactivated modified enzyme even after the secondary injection. IgG responses were also smaller following immunization with the modified enzymes, but these responses would eventually reach levels comparable to those obtained with the native enzyme.

DISCUSSION

If our animal experimental results can be extended to the clinical setting, the following observations should be made:

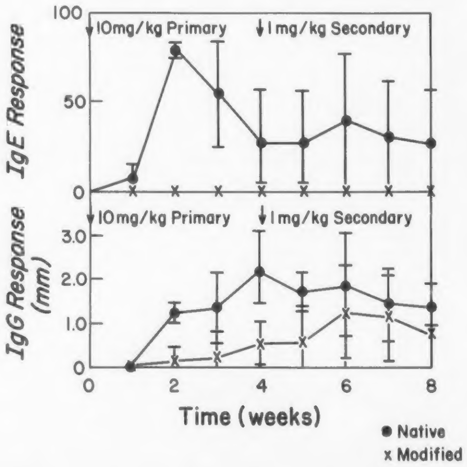


FIGURE 8.—Radial immunodiffusion diameters (IgG response) and PCA titers (IgE response) of serum from mice immunized with DONV inactivated native or poly-D,L-alanyl modified *Erwinia* L-asparaginase in complete Freund's adjuvant. IgE content of serum was measured by PAC in rats. An intradermal injection of 0.1 ml of serum diluted 10–80-fold was given 48 hours prior to an intravenous challenge injection of 125 μg homologous antigen in 1 ml 0.5% Evans Blue dye. Rats were killed after 30 minutes and the diameter of the extravasated dye was measured on the underside of the skin. Results were recorded as the reciprocal of the highest dilution giving a positive reaction. IgG content of serum was measured by reversed single radial immunodiffusion. Native enzyme (62.5 μg/ml) was incorporated in Agarose gel and undiluted serum was introduced in the wells. The diameter of the precipitated rings was measured after 14–16 hours at room temperature.

First, the longer plasma half-life of the modified enzyme may mean that smaller dosages are required for an equivalent therapeutic response.

Second, the delayed appearance of IgG antibodies against the modified as opposed to the native enzyme may prevent the immune-related drug clearance phenomenon, particularly since the enzyme would not be given as an inactive protein with complete Freund's adjuvant.

Third, the therapy-limiting hypersensitive reaction should be greatly reduced by employing the modified enzymes since, under our experimental conditions, these preparations have not evoked an IgE response.

It is also worth considering the possible value of the modified enzymes as an adjunct to, rather than a replacement for, therapy with the native enzymes. The 300- to 500-fold lower cross-reactivity between the native and modified forms implies that one may be able to treat patients who have low antiasparaginase titers with modified forms of the enzyme. Liu and Katz have recently reported that the attachment of D-glutamate-lysine copolymers to both ovalbumin and ragweed antigen not only prevents IgE formation to the modified antigen but also induces tolerance to the native antigen (21). We are currently investigating whether or not our modified forms of asparaginase will also induce tolerance to the native enzymes.

Finally, L-asparaginase therapy combined with better inhibitors of asparagine synthetase may eliminate host liver sanctuaries for asparaginase-sensitive tumor cells and possibly broaden the spectrum of tumors that respond to such treatment.

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L-Asparaginase: Human Toxicology and Single Agent Activity in Nonleukemic Neoplasms

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SUMMARY

The early hope that L-asparaginase would be a breakthrough in medical treatment, with selective toxic effects based on the qualitative presence or absence of a specific enzyme (asparagine synthetase), has not been realized. Despite its failure to live up to early hopes, L-asparaginase is now commercially available because of its usefulness in treating selected forms of acute leukemia and T-cell lymphoid neoplasms. By and large, hints of useful activity in other tumors have not been confirmed, and L-asparaginase remains experimental for all other indications. It is variably toxic in man, and severe toxic effects are not unusual. Toxic reactions are generally hypersensitivity reactions or depression of protein synthesis.

[Cancer Treat Rep 65 (Suppl 4):57-59, 1981]

Capizzi (1) has described the natural history of newly discovered drugs as beginning with a period of great enthusiasm while the number of patients treated is very small, followed by loss of enthusiasm as more patients are treated and new toxic effects are demonstrated. This may be followed by a latent period, during which the drug appears to be abandoned while ongoing research is conducted, followed by a final period in which the drug is placed in its proper therapeutic perspective and its use is described in standard medical textbooks. Clearly, this describes the history of the clinical use of L-asparaginase. The first reports of the use of this drug in man were those of Hill et al (2) and Oettgen et al (3) in 1967. Initially, the drug appeared to be quite nontoxic and to be highly active against human leukemia. However, toxic reactions began to be described over the next several years, and reports from the National Cancer Institute (NCI) in 1969 clearly established a wide spectrum of toxic reactions to this agent (4,5). L-Asparaginase remained of great interest despite these toxic reactions, although it soon became clear that the duration of response in acute leukemia was extremely short and that the therapeutic index of the drug was low. Following this flurry of activity, which included identification of L-asparaginase by *Time* magazine in 1970 as one of the "breakthroughs" of the 1960s, the drug went into relative eclipse. However, it has survivied this latent period and is now an established drug for the treatment of acute lymphoblastic leukemia.

This brief review will summarize the toxic effects of L-asparaginase as well as its single-agent activity in nonleukemic neoplasms. More details are available in several excellent reviews (6-8), as well as in selected original clinical reports from the NCI (5), Memorial Sloan-Kettering Cancer Institute (9-11), Yale (12), Southwest Cancer Chemotherapy Study Group (13), Acute Leukemia Group B (14), Harvard (15), and London (16).

TOXIC EFFECTS OF L-ASPARAGINASE

Unlike most other antineoplastic agents, L-asparaginase is not usually toxic to bone marrow stem cells, oral and gut mucosa, or hair follicles. However, it may have severe effects on the liver, kidneys, pancreas, central nervous system, immune system, and clotting system. The toxic effects of L-asparaginase are mainly hypersensitivity reactions and inhibition of protein synthesis. These are separable from the toxicity of endotoxin that may contaminate *Escherichia coli* enzyme preparations. The form and severity of toxicity vary considerably from patient to patient, and this variation is not simply explained by analyzing the dose schedule or route of administration

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employed. It appears that pharmacogenetic differences in L-asparaginase tolerance must exist, probably due to individual differences in the ability of target cells to synthesize L-asparagine.

Following L-asparaginase administration, the majority of patients develop anorexia, nausea, vomiting, and low-grade fever. Occasionally, these are severe enough to modify therapy, and, rarely, hyperpyrexia may be life-threatening or even fatal. Hypersensitivity reactions are common with the symptoms usually developing many days or weeks after the initial treatment. Occasionally, hypersensitivity reactions are limited to urticaria, although hypotension, moderate respiratory distress with wheezing, and, rarely, a serum sickness-like reaction with swollen joints and sterile effusion may be seen. Anaphylactic shock has also occurred and is not prevented by the concomitant use of corticosteroids. The recent use of an im route for L-asparaginase appears to have decreased the frequency of life-threatening anaphylactic shock (17), but it nevertheless remains a problem. Indeed, anaphylaxis has been reported to be especially common in patients receiving L-asparaginase iv soon after concomitant treatment with vincristine and prednisone (Asparaginase Product Brochure, Merck, Sharp & Dohme, November 1977). Because of this, all patients receiving the drug should be carefully observed for the development of anaphylaxis and promptly treated with epinephrine as needed. Rarely, other supportive therapy including intubation may be necessary. The development of anaphylaxis usually precludes repeated courses of treatment with L-asparaginase, although re-treatment is occasionally possible with immunologically unrelated L-asparaginase preparations or following desensitization.

Liver dysfunction occurs in most patients treated. It is primarily manifested as a decrease in serum albumin, elevated transaminases, elevated alkaline phosphatase, decreased serum cholesterol, decreased lipoprotein synthesis, and hyperbilirubinemia. Liver biopsy usually shows fatty metamorphosis. Decreased levels of clotting factors V, VII, VIII, IX, prothrombin, and fibrinogen may also be seen, almost always because of decreased clotting factor synthesis (18). Despite the coagulation factor abnormalities, bleeding is rare. In general, liver dysfunction appears to be secondary to protein synthesis inhibition, and it is usually quickly reversed by discontinuing the drug. This reduction in protein synthesis may complicate the interpretation of tests unrelated to liver function. For example, L-asparaginase may induce an acute, asymptomatic reduction in the level of thyroxine-binding globulin (19).

Both the exocrine and endocrine function of the pancreas may be affected by L-asparaginase. Severe pancreatitis has occurred, including deaths from hemorrhagic pancreatitis. Less extensive signs and symptoms of pancreatitis may occur with a rise in serum amylase and lipase. Malabsorption may also occur (20). In general, pre-existing pancreatitis should be considered a contraindication to the use of the drug.

Hyperosmotic, nonketotic hyperglycemia has been reported, and this usually responds to insulin and hydration. Low levels of insulin have been reported during episodes of hyperglycemia caused by L-asparaginase, presumably due to decreased insulin synthesis (21). However, more recent studies suggest that defective insulin release (22) or a poorly explained decrease in insulin receptors (23) may also occur with L-asparaginase treatment.

Most patients develop an elevated BUN, which usually represents prerenal azotemia. Serum creatinine, creatinine clearance, and urinary sediment measurements are usually normal, although rare patients may develop renal failure with marked oliguria. Uric acid nephropathy is usually not a problem in patients who receive adequate hydration and allopurinol.

Central nervous system dysfunction is common in adults receiving L-asparaginase but is rare in children. The symptoms and signs range from mild depression and drowsiness to impaired sensorium, coma, and an acute organic brain syndrome (4,7,24). Abnormal electroencephalograms have been reported. The CNS dysfunction generally resolves rapidly following discontinuation of L-asparaginase therapy. The mechanism of these CNS abnormalities is unknown, although it has been postulated that it is related to modifications of L-glutamine levels due to glutaminase contamination of the L-asparaginase preparation, and in some cases high concentrations of ammonia in the blood have been noted.

L-Asparaginase may affect the immune system in two ways: hypersensitivity reactions may occur as may immunodeficiency (25). The latter appears to occur primarily through inhibition of blastogenesis by thymus-dependent (T-cell) lymphocytes, although under certain circumstances B cells are also inhibited (26). The immunosuppressive effects of L-asparaginase have occasionally been employed for clinical immunosuppressive therapy after renal or bone marrow transplantation.

The use of L-asparaginase during pregnancy has not been reported, although its use in pregnant rabbits leads to fetal resorptions and malformations (27).

In general, toxic indications for discontinuing L-asparaginase therapy include anaphylaxis, pancreatitis, markedly decreased protein synthesis, a severe organic brain syndrome, or marked interference with glucose metabolism.

SINGLE-AGENT ACTIVITY IN NONLEUKEMIC NEOPLASMS

The only tumors known to respond to L-asparaginase therapy with any reproducible frequency are lymphoid neoplasms, primarily of T or null lymphocyte origin. These include lymphoblastic transformation of chronic granulocytic leukemia, the T or null cell lymphoblastic lymphoma associated with an elevated terminal transferase content and a high frequency of mediastinal masses, as well as the usual form of acute lymphoblastic leukemia in children.

Although rare cases of malignant melanoma have shown transient responses, the bulk of evidence favors no role for L-asparaginase in the treatment of this disease (5,10,28).

Two cell lines of pancreatic adenocarcinomas in tissue culture have proven sensitive to L-asparaginase (29), but human trials have shown no objective evidence of benefit in patients with adenocarcinoma of the pancreas (30). Anecdotal benefit from L-asparaginase has been reported for patients with insulinomas (31,32), but the role of L-asparaginase in these patients remains undefined.

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Enzyme Therapy of Cancer, Future Studies[1]

J.S. Holcenberg[2,*]

SUMMARY

The dramatic clinical responses to L-asparaginase led to renewed interest in other enzymes that might be effective antitumor agents. Biochemical and nutritional studies of animal and human tumors have shown that enzymatic depletion of glutamine, arginine, cysteine, citrulline, and serine could have selective cytotoxicity for some tumors. Several glutaminase-asparaginase enzymes have antitumor activity in animals and man. These enzymes are currently in phase I trials. Arginine-depleting enzymes with suitable properties for therapy have been developed and are in preclinical study. Enzymes have not yet been found that can adequately deplete circulating levels of cysteine, citrulline, or serine for treatment of cancer.

[Cancer Treat Rep 65 (Suppl 4):61–71, 1981]

This symposium has presented the preclinical development and clinical effectiveness of L-asparaginase as a cancer chemotherapeutic agent. Asparaginase is an especially exciting drug because its cytotoxicity is based on a biochemical difference between sensitive and resistant cells. Asparagine is an essential amino acid for asparaginase-sensitive cells. Under normal conditions, asparagine does not have to be synthesized by cells since it is readily available in the diet. Sensitive cells are killed only when the circulating level of asparagine is depleted by enzyme therapy. Since most normal cells can synthesize asparagine, this therapy should be highly selective of tumor cells that have lost this capacity. The clinical effectiveness of asparaginase is limited by its antigenicity, toxicity, nonspecificity, and limited spectrum of antitumor activity.

Asparaginase enzymes are foreign proteins that evoke an immune response characterized by acute allergic reactions and loss of enzyme activity. This problem can be minimized by combination therapy with immunosuppressant drugs or by the enzyme modifications discussed by Dr. Uren.

Asparaginase toxicity is not very specific for tumor cells since it can decrease protein synthesis throughout the body and can cause severe pancreatic and central nervous system toxicity. The asparaginase enzymes in clinical use not only deplete asparagine but also partially deplete glutamine levels. Some of the toxicity may be due to this lack of specificity. Asparaginase enzymes without glutaminase activity can be purified from the serum of some animals and from bacteria. None of these pure asparaginase enzymes have been sufficiently tested to evaluate their toxicity.

The lack of specificity of the current enzymes can potentially decrease the antitumor activity towards asparaginase-sensitive tumors. In enzymes that can hydrolyze both asparagine and glutamine, both amino acids compete for the active site of the enzyme. Since extracellular fluid, plasma and tissue culture media contain 10 to 100 times more glutamine than asparagine, enzymes with both activities have relatively less asparaginase activity than specific asparaginase enzymes. Figure 1 illustrates this problem with a simulation of the effect of two enzymes on the asparagine levels in tissue culture media. *Escherichia coli* asparaginase (EC-2) has much less competing glutaminase activity than *Acinetobacter* glutaminase-asparaginase (AGA). For this reason, the concentration of asparaginase activity needed to deplete asparagine levels is ten times lower with EC-2 than with AGA. Cytotoxicity experiments involving asparaginase-sensitive L5178Y mouse leukemic cells showed this same difference since the cells were killed by much lower doses of EC-2 than AGA (1). An asparaginase enzyme without any glutaminase activity would probably have even greater activity than EC-2.

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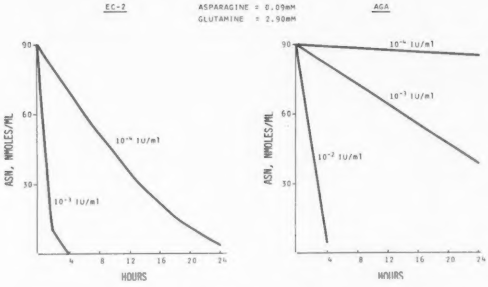

FIGURE 1.—Simulation of asparagine concentration in tissue culture media treated with various concentrations of *E. coli* asparaginase (EC-2) and *Acinetobacter* glutaminase-asparaginase (AGA). Initial concentrations of asparagine and glutamine were .09 and 2.9 mM, respectively. Results were computed by Michaelis-Menton kinetics for an enzyme with two competing substrates. The kinetic properties of the enzymes are listed in table 2.

Another major problem has been the limited number of tumors that respond to asparaginase therapy. Combination therapy as discussed by Dr. Capizzi appears to increase the number of sensitive tumors.

We have sought other amino-acid-degrading enzymes to increase the spectrum of enzyme therapy. This approach is based on the expectation that certain tumor cells lack the enzymes necessary to synthesize other nonessential amino acids. This enzyme defect would be inapparent because of the high concentration of nonessential amino acids in body fluids. Enzyme therapy that depletes the amino acid would unmask this requirement and selectively kill the tumor cell. All nonessential amino acids are candidates for this approach. The most promising are glutamine, arginine, citrulline, and serine since some tumor cells have increased requirements and defective biosynthetic activity for these amino acids (2–4).

Several glutaminase and glutaminase-asparaginase enzymes have been purified, characterized, and tested for antitumor activity (4,5). The most promising glutaminase-asparaginase enzymes are from *Acinetobacter*, *Pseudomonas* and *Achromobacter* species. The properties of the first two enzymes are compared with *E. coli* and *Erwinia* asparaginase in table 1. All four enzymes have similar size and kinetic properties for hydrolysis of asparagine. They differ greatly in their Km for glutamine and ratio of glutaminase to asparaginase activity. The *Pseudomonas 7A* enzyme has a much longer plasma half-life than any of the other enzymes and has greater antitumor activity in animals than the *Acinetobacter* glutaminase-asparaginase. Both glutaminase-asparaginase enzymes have antitumor activity against asparaginase-resistant tumors (6). *Achromobacter* and *Acinetobacter* glutaminase-asparaginase have very similar properties (7).

Figure 2 illustrates the importance of Km and relative activity for depletion of glutamine. The simulated glutamine levels and glutaminase activity are plotted at various times after a single identical dose of asparaginase activity with EC-2 and AGA. Because of its lower Km for glutamine and higher relative activity, AGA produces a much greater and longer depletion of glutamine than does EC-2. Both enzymes produce a similar depletion of asparagine. These predictions have been confirmed in animal studies. Thus, a low glutamine Km is essential for effective glutamine depletion.

Human trials with glutaminase-asparaginase were first conducted by Spiers and Wade with the *Achromobacter* enzyme (7). Nine patients were treated with more than one dose of the enzyme. The enzyme had a plasma half-life of only 80 minutes. This short half-life

TABLE 1.—Properties of therapeutic enzymes*

Enzyme	Km, μM		Ratio maximal activity	Molecular weight	pI†	Plasma t1/2 mice	
	L-GLN	L-ASN	L-GLN/L-ASN			LDHV-‡	LDHV+‡
Acinetobacter GA	5.8	4.8	1.2	138,000	8.2	1 hr	13–18 hr
Pseudomonas GA	4.6	4.4	2.0	139,000	5.8	13	43
E. coli A	3000	12	0.03	141,000	5.0	2–4	20–30
Erwinia A	1000	10	0.05	138,000	8.7	3–4	24

*Values in this table obtained from the literature. GA and A represent glutaminase-asparaginase and asparaginase.

†Isoelectric point.

‡LDHV- and + indicate mice infected or uninfected with lactate dihydrogenase elevating virus that is present in most transplantable mouse tumors.

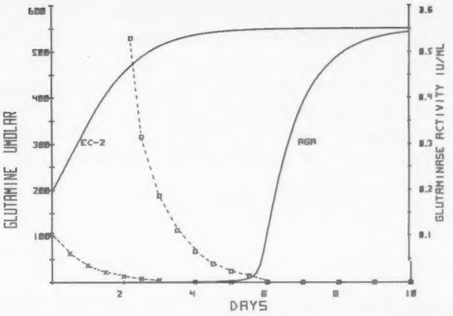

FIGURE 2.—Simulation of glutamine concentration and glutaminase activity in plasma of mice treated with identical asparaginase doses of *E. coli* asparaginase (EC-2) or *Acinetobacter* glutaminase-asparaginase (AGA). Initial asparaginase activity was 3.5 IU/ml. Plasma half-life of both enzymes was assumed to be 18 hrs. Glutaminase activity is shown by dashed lines (X for EC-2 and ☐ for AGA). Glutamine levels are shown by solid lines and were calculated by the equation in references 4 and 15. The maximum rate of glutamine input into plasma was assumed to be 0.014 μmoles/ml/minute.

necessitated continuous infusions of 15,000 to 20,000 IU/m^2/day for persistent depletion of asparagine and glutamine levels. Two patients with ALL and four with AML were treated with these infusions for 3 to 28 days. The blast count fell in five patients and was undetectable in two patients. Spleen size and the percentage of marrow blasts decreased in some patients, but no remissions were achieved. Fifteen additional patients with ALL were treated with this enzyme and methotrexate in a regimen similar to the one described by Dr. Capizzi for L-asparaginase (8). Two complete and three partial remissions were achieved. Current studies are using preparations made by polymerization or covalent attachment to polyethylene glycol since these appear to have longer plasma half-lives (9).

We have shown that treatment of *Acinetobacter* glutaminase-asparaginase with succinic anhydride increases its half-life without changing its catalytic properties (10). A protocol was implemented at the Midwest Children's Cancer Center to test the human pharmacology, toxicity, and antitumor activity of succinylated AGA. AGA was purified and succinylated in our laboratory. It was administered in daily doses of 200–2000 IU/m^2 for up to 34 days to 21 children with refractory leukemia or solid tumors. The enzyme preparations had an isoelectric point of 5.1 to 5.7 and a specific activity of 117 to 150 IU/mg protein. The enzyme activity of the succinylated preparations in plasma decreased with a half-life of 27.4 ± 7.4 hrs. The native enzyme had a half-life of only 1 hour. Doses of 200 IU/m^2 or greater reduced the plasma glutamine and asparagine to undetectable levels ($<2\,\mu M$) for more than three days. The enzyme activity and levels of aspartate and glutamate in plasma increased with the dose. Ammonia levels increased in all patients but did not correlate with glutaminase or glutamate levels. Urinary excretion of glutamate increased an average of 3000-fold, indicating that the high filtered load of glutamate exceeds the renal resorptive capacity. Cerebrospinal fluid levels of glutamine increased despite the depletion of this amino acid in plasma. Enzyme activity was detectable in the cerebrospinal fluid of five of eight patients tested; the average activity was 0.5% of the plasma level. Pleural fluid levels of glutaminase reached about 15% of plasma level after three daily intravenous doses (11,12).

These studies show that succinylated AGA has the same distribution and plasma half-life as *E. coli* and *Erwinia* asparaginase. Succinylated AGA differs from the asparaginase enzymes in its effect on amino acid levels in body fluids. Asparaginase produces prolonged depletion of plasma asparagine, transient depletion of plasma glutamine, marked elevation of ammonia, three- to fourfold elevation of glutamate, and little effect on other amino acids (2–4). Succinylated AGA produces prolonged depletion of both asparagine and glutamine, similar elevation of ammonia, and much greater elevation of glutamate, aspartate, and threonine levels.

Toxic effects were similar to those described with *E. coli* asparaginase and *Achromobacter* glutaminase-asparaginase (table 2). Nausea, vomiting, and anorexia occurred in 14 patients, transient fever in nine, hypoalbuminemia or hypofibrinoglycemia in six, and hyperglycemia without acidosis in seven. Insulin levels were very low in the four patients tested during hyperglycemia. A mild respiratory alkalosis was seen in 11 patients. Calcium decreased in half of the patients studied. No abnormalities were seen in urinalysis, renal function, serum amylase, or bilirubin. Myelosuppression was difficult to evaluate in these children because of extensive prior chemotherapy or radiotherapy and bone marrow replacement by tumor. Two patients without bone marrow disease had no myelosuppres-

TABLE 2.—Toxic effects of succinylated *Acinetobacter* AGA

	No. abnormal/No. evaluated
Nausea, vomiting, anorexia	14/20
Transient fever	9/20
Glucose >160 mg/dl	7/20
Albumin <2.8 g/dl	5/20
Fibrinogen <200 mg/dl	6/18
Cholesterol <150 mg/dl	10/14
pH >7.48	11/17
Total CO_2 <21 mEq/liter	19/20
Calcium <8.9 mg/dl	8/15

sion. In contrast, granulocyte, platelet, and reticulocyte counts decreased in most patients with marrow disease.

The most severe toxic effects were somnolence and coma that occurred in five patients. Of these patients, two had central nervous system leukemia that progressed during enzyme therapy. This progression may have been due to the elevated glutamine levels in the cerebrospinal fluid. A third patient developed coma as a terminal event after hyperglycemia and a severe gastrointestinal hemorrhage. These three patients died. Another patient developed transient coma only during septicemia and a rapid rise in lymphoblast count. He recovered completely in 2 days with treatment of the infection and leukemia. The fifth patient had prior central nervous system leukemia, cranial irradiation, and extensive intrathecal methotrexate. She became unresponsive after only two 1500 IU/m^2 doses of the enzyme. With supportive care, she awoke in 5 days and appeared normal 2 days later. Since this experience we have not treated any patients with concurrent or prior central nervous system leukemia. Of the last seven patients, none has become somnolent. Two patients had acute allergic reactions characterized by rash, fever, and chills. No respiratory distress was seen. These allergic reactions occurred after 16 and 20 doses of the enzyme.

Treatment with succinylated AGA decreased the blast count by greater than 90 percent in eight of 15 children with acute lymphocytic leukemia and in the only patient with acute myelogenous leukemia (table 3). Lymphoblasts were eliminated from the blood of five of these patients. Three patients developed bone marrow aplasia followed by partial bone marrow remissions of short duration. A patient with B-cell leukemia and one with lymphoma had greater than a 50% decrease in measurable masses during therapy. No response was seen in individual patients with Ewing's sarcoma and ovarian teratocarcinoma.

Clinical trials of succinylated AGA are currently underway at the Memorial Sloan-Kettering Cancer Center. Children and adults with refractory tumors are being tested on daily and intermittent schedules. In our institution, we are currently testing the effect of amino acid supplementation on the toxic and antitumor effects of succinylated AGA.

CONCLUSION

These early studies show that glutaminase enzymes have great potential to extend the spectrum of human tumors that can be treated by enzyme therapy. Much additional work is needed to develop these new antitumor agents. First, further studies are needed of the glutaminase-asparaginase enzymes with long plasma half-lives. These include native *Pseudomonas 7a*, succinylated *Acinetobacter*, and polyglycolated *Achromobacter* glutaminase-asparaginase. Second, these enzymes should be tested in combination with amino acid antagonists like diazo-oxo-norleucine (DON), AT125 (6,13), and other antitumor agents like melphalan and methotrexate (14). Third, preclinical studies are needed to evaluate the toxic effects and antitumor action of specific asparaginase and glutaminase enzymes. Theoretically, these enzymes should be more active and less toxic. Finally, additional study is needed to develop enzymes that can degrade arginine, cysteine, citrulline and serine. Certain tumor cells have much greater requirements for these amino acids than do normal tissues. Arginine-degrading enzymes with suitable properties are available and are currently undergoing preclinical testing. Currently, there are no enzymes with sufficient activity under physiologic conditions for enzyme depletion of the

TABLE 3.—Patient summary

Diagnosis	No.	Age range	Sex	Response to therapy*	
ALL, non T-non B	13	3–17	10 M	7/12	2 PR†
ALL, T cell	2	14	1 M	1/12	1 PR
ALL, B cell	2	10, 11	2 M	0/1	1 PR
Acute myelogenous leukemia	1	4	1 F	1/1	
Solid tumors‡	3	6-16	1M	1/3	1 PR

*Response to therapy is defined as a decrease in lymphoblasts or myeloblasts in blood of greater than 1 $\log_{10}$. Fraction shown is number responding/number of patients with blasts in the blood. A partial response was defined as a bone marrow with 5% to 25% lymphoblasts plus stem cells in leukemia, or a decrease in measurable masses of greater than 50% in solid tumors. The partial response occurred in the lymphoepithelioma.

†PR = partial response.

‡One each: Ewing's sarcoma, lymphoepithelioma, and ovarian teratocarcinoma.

other amino acids. Antimetabolites of these amino acids have been developed and are being studied in animals.

Thus, the experience with L-asparaginase has led to the development of a potentially very effective therapy directed toward unusual requirements for other nonessential amino acids by tumor cells.

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Sequential Studies of Daunorubicin and Adriamycin in Acute Nonlymphocytic Leukemia

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Daunorubicin was first demonstrated to be a highly active drug for remission induction of acute nonlymphocytic leukemia (ANLL) by Bernard et al (1) in 1967. In this country significant antileukemic activity for daunorubicin was first confirmed by Cancer and Leukemia Group B (CALGB), formerly the Acute Leukemia Group B (2). A 43% complete response rate in previously untreated adults with ANLL was achieved with daunorubicin 60 mg/M^2 given daily for 5 days. The first comparative trials with this agent were performed at the Baltimore Cancer Research Program (BCRP) of the Division of Cancer Treatment, NCI. Initial BCRP studies (3) used a dose of 60 mg/M^2 given daily for 3 days and demonstrated that treatment with daunorubicin alone resulted in a complete response rate (50%) far superior to the best treatment of the day, the POMP (prednisone, vincristine, methotrexate, and 6-mercaptopurine) regimen (28%). A second study conducted at the BCRP failed to demonstrate superiority for a daunorubicin-containing four-drug combination compared to daunorubicin alone, and a similar study conducted by CALGB confirmed these data (4,5). Table 1 summarizes these early data.

Yates et al (6) reported in 1973 that a continuous 7-day infusion of cytosine arabinoside, 100 mg/M^2/day, in association with three daily doses of daunorubicin, 45 mg/M^2 daily for 3 days, yielded a greater complete response rate (67%) than that reported for daunorubicin alone. CALGB (7) confirmed the efficacy of that regimen by achieving an identical response rate and, while no direct comparison to daunorubicin alone was made, the group did demonstrate the superiority of that regimen compared to less intensive treatments (fig 1). A recent BCRP study (8) demonstrated that the Yates and Holland regimen is superior to daunorubicin alone for induction therapy of ANLL even when patients are selected for the single-drug regimen on the basis of certain leukemic cell biochemical criteria (fig 2). The combination resulted in a 60% complete response rate compared to 42% for the single drug. The Yates and Holland regimen, commonly known as the "7 and 3" regimen, has been widely accepted as one of the most efficacious induction treatments devised to date (table 2).

Recently several investigators have attempted to improve that regimen by adding thioguanine to it (table 3). The results of some studies suggest improved efficacy with the three drugs (9,10). However, in the largest such study which was conducted by CALGB, the three-drug combination offered little advantage over treatment with daunorubicin alone except for patients less than 20 years of age (5,11). That latter group of patients had a significantly higher complete response rate (90%) with the three-drug compared to the single-drug treatment (63%), but the "7 and 3" regimen was not tested in that study. A new CALGB study (Protocol 7921) under the direction of Dr. Harvey Preisler will directly compare the remission induction capability of the "7 and 3" regimen with a daunorubicin, cytosine arabinoside, and thioguanine regimen.

Modern treatments such as the "7 and 3" regimen that utilize smaller doses of daunorubicin in conjunction with other agents have generally yielded maximum benefit in responding patients after one treatment course. Since most patients who will respond do so after having received only approximately 25%-50% of the maximum "cardiac safe" dose of daunorubicin (< 550 mg/M^2), such patients are candidates for studies of daunorubicin during the maintenance phase of treatment. Early evaluation of some such studies reveals enhanced remission duration for patients receiving daunorubicin intermittently during remission compared historically to patients who received no daunorubicin during remission (12). The large number of uncontrolled variables at play in such a comparison dictate caution in interpretation, however. A recently completed CALGB study directed by Dr. Jerome Yates[2] shows no difference in response duration to date in patients who received daunorubicin intermittently during remission compared to those who received it twice as often. Therefore, the value of daunorubicin administered during remission in patients with ANLL must be considered unproven at this time.

Studies at M.D. Anderson Hospital (14) and at Roswell Park Memorial Institute (15) have demon-

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[2] Yates JW. Personal communication, 1980.

TABLE 1.—Early American data on daunorubicin in ANLL

Investigator* and year of report	Dose and schedule of DNR	No. patients	% CR	Maintenance therapy†	Duration CR (mo.)	Ref
CALGB, 1973	60 mg/M²/day × 5	39	43	6MP + MTX or Ara-C + MeGAG	4.4	2
BCRP, 1972	60 mg/M²/day × 3	22	50	None	2.3	3
BCRP, 1976	Same	33	49	Half dose DNR then monthly CTX + GNZ	6.8	4
CALGB, 1979	Same	144	42	Monthly CTX + HU, then 6MP, 6TG, or both	5.4	5

*CALGB = Cancer and Leukemia Group B, BCRP = Baltimore Cancer Research Program, DCT, NCI.

†6MP = 6-mercaptopurine, MTX = methotrexate, Ara-C = cytosine arabinoside, MeGAG = methyl GAG, DNR =daunorubicin, CTX = cyclophosphamide, GNZ = guanazole, HU = hydroxyurea.

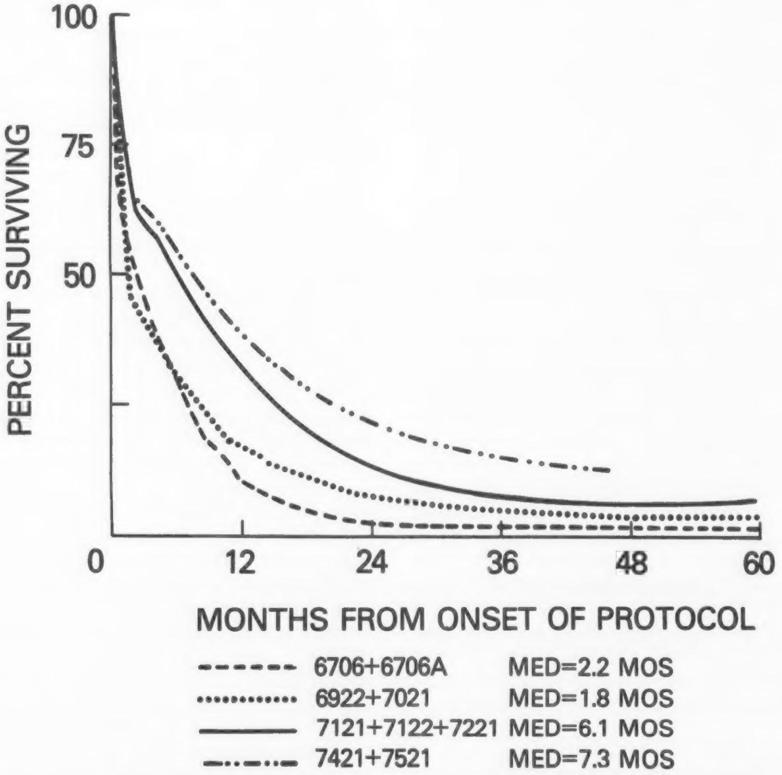

FIGURE 1.—Survival curves for all treated patients on serial CALGB studies on ANLL. The various treatment options in protocols 7121, 7122, and 7221 included daunorubicin alone or in combination with other agents in moderately intensive regimens. The majority of patients in protocols 7421 and 7521 were treated with the "7 and 3" regimen of daunorubicin 45 mg/M² daily, Days 1-3 and a continuous iv infusion of cytosine arabinoside Days 1-7 at the rate of 100 mg/M²/day.

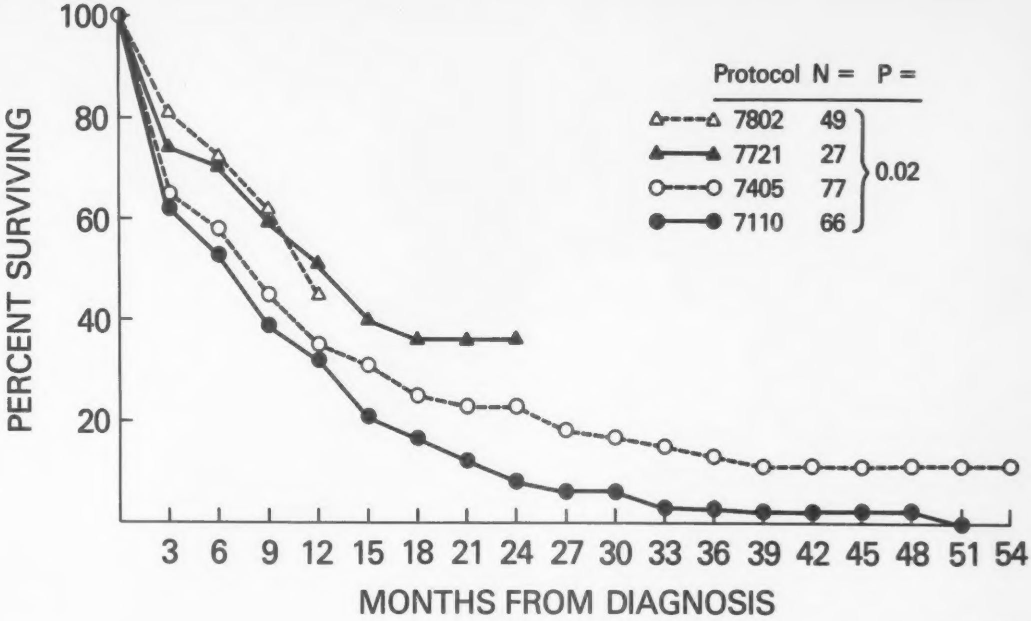

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FIGURE 2.—Survival curves for all treated patients on serial BCRP studies of ANLL. Studies 7405, 7721, and 7802 utilized the "7 and 3" regimen for induction therapy primarily, and tested several different maintenance options. In 7110 half the patients were treated with daunorubicin alone and half were treated with a moderately intensive regimen which included daunorubicin. The same trend toward improved median and long-term survival demonstrated in figure 1 is seen here also.

TABLE 2.—Results in ANLL with the "7 and 3" regimen*

Investigator† and year of report	No. patients	% CR	Ref
Yates et al, 1973	9	67	6
CALGB, 1975	43	66	7
BCRP, 1979	60	60	8

*Daunorubicin 45 mg/m^2/day Days 1–3 with a continuous iv infusion of cytosine arabinoside 100 mg/M^2/day, Days 1–7.

†See footnote to table 1.

strated the efficacy of Adriamycin in the treatment of ANLL. Those studies have suggested that smaller doses of Adriamycin may be as active as larger daunorubicin doses. However, a recently completed CALGB study demonstrates that the drugs are equally active when each is used in low dose (30 mg/M^2 daily × 3) in conjunction with cytosine arabinoside in a "7 and 3" schedule (13). While both drugs may be equally active, it may be more difficult to use Adriamycin than daunorubicin. Studies at the BCRP have demonstrated a significantly greater excretion of Adriamycin than daunorubicin by the liver (16,17). The clinical importance of this observation is that Adriamycin doses must be substantially reduced in the presence of any hepatic excretory dysfunction in order to avoid undue drug toxicity, whereas daunorubicin can almost always

Table 3.—Results in ANLL with daunorubicin, cytosine arabinoside, and thioguanine

Investigator*	Regimen†	No. patients	% CR	Ref
BCRP, 1976	DNR 75 mg/M^2 Day 1 Ara-C 75 mg/M^2 q 12 h Days 1–5 GTG 75 mg/M^2 q 12 h Days 1–5	33	46	4
CALGB, 1979	DNR 100 mg/M^2 Day 1 Ara-C and 6-TG each 100 mg/M^2 q 12 h Days 1–5	154	50	5
Gale, 1977	DNR 60 mg/M^2/day, Days 5–7 Ara-C and 6-TG each 100 mg/M^2 q 12 h, Days 1–7	28	79	9
Rees, 1977	DNR 50 mg/M^2 Day 1 Ara-C and 6-TG each 100 mg/M^2 q 12 h, Days 1–5	20	85	10

*See footnote to table 1.

†See footnote to table 1.

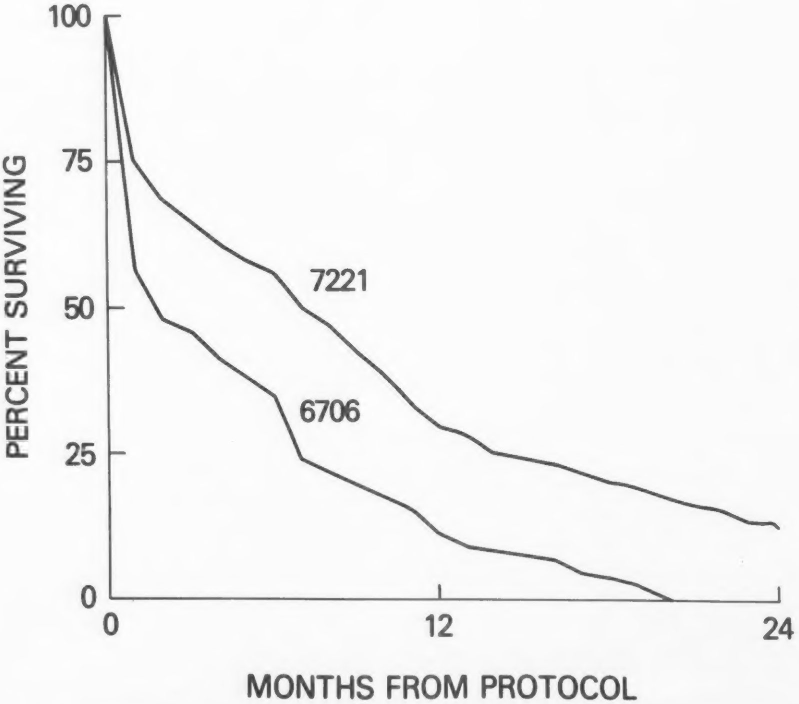

Figure 3.—Two groups of CALGB patients treated with the same dose and schedule of daunorubicin alone. See text for details.

be given safely in full dose except in the presence of severe liver dysfunction. Failure to recognize this fact can lead to unexpectedly prolonged plasma Adriamycin levels which may result in severe oral mucositis and other gastrointestinal toxicity including, rarely, perforation (18).

Since there appears to be no advantage for Adriamycin over daunorubicin in the treatment of ANLL, and because daunorubicin may be more safely administered at the anthracycline doses required for optimal activity, we prefer daunorubicin over Adriamycin for the treatment of ANLL. The choice of daunorubicin may be especially important in some variants of ANLL such as promyelocytic leukemia and erythroleukemia which have been demonstrated to be especially sensitive to that drug (19,20). Similar data for Adriamycin in these variants have not been reported.

In the 18 years that have elapsed since the isolation and purification of daunorubicin, multinational research has identified this compound as the most active drug for the treatment of ANLL. Recently combinations of drugs which include daunorubicin have been devised that are clearly superior to daunorubicin alone for the treatment of this disease. Future research will delineate the role, if any, of daunorubicin in the remission phase of the disease and, with hope, will yield even more effective daunorubicin-based combination treatments. Analog research could result in less toxic compounds which retain the activity of daunorubicin.

One cautionary note with respect to evaluating sequential studies in this disease: a variety of supportive techniques have been improved over the years that may have a favorable effect on the outcome of any study. Figure 3 illustrates the survival curves for all treated patients who received only daunorubicin (same dose and schedule) as induction therapy on two CALGB studies. Study 6706 began in 1967 and Study 7221 began in 1972. Although the curves are widely divergent they are, in fact, parallel except for the first few months of observation. Approximately 45% of patients in study 6706 died shortly after treatment whereas only 25% of patients in 7221 were early deaths. The difference in the two curves results primarily from improved supportive care available during the second study. If 6706 were drug "A" and used as an historical control for a different, new treatment, drug "B" (7221), we might come away with the impression that drug "B" was superior to drug "A." It is for this reason that I feel that randomized prospective studies with concurrent controls are necessary to assess properly the results of treatment research in this disease.

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Anthracyclines in the Treatment of Acute Nonlymphocytic Leukemias[1]

Michel Boiron,[2,*] Claude Jacquillat,[2] Michel Marty,[2] Marise Weil,[2] and Christian Gisselbrecht[2]

The isolation in 1962 by Dubost et al (1) of the anthracycline antibiotic daunomycin (DNR) generated a special interest in the chemical, biological, and clinical studies of anthracyclines; indeed, they have been one of the most significant discoveries in cancer chemotherapy in the last 15 years. Several hundred natural or semi-synthetic derivatives have now been studied, of which several have led to special interest in anticancer chemotherapy.

Amongst these derivatives, daunomycin, daunorubicinol, rubidazone, and more recently detorubicin have been studied since 1966 in more than 5000 patients in the Department of Hematology in Hôpital Saint Louis, Paris. This paper will summarize main data obtained from these studies in patients with acute nonlymphocytic leukemias.

Single Agent Chemotherapy with Anthracycline in ANLL

This type of regimen allows for objective evaluation of the antileukemic value of the different compounds as well as for evaluation of acute, subacute and chronic toxicity in man.

We shall refer to different subtypes of ANLL according to the FAB classification (2).

Daunomycin

Rubidomycin or daunomycin (DNR) has been the first anthracyclic compound that we used as a single agent in acute leukemias. When DNR was introduced in the treatment of ANLL, complete remission rates achieved with combinations including AraC and/or 6-mercaptopurine (6MP) and/or *bis*-methyl-guanylhydrazone, hardly reached 40% (3).

Forty-two patients were included in our first study with DNR (4). DNR was administered daily (2 mg/kg) until bone marrow was cleared from leukemic cells. Twenty-two patients (51%) achieved complete remission. The mean dose to complete remission was 11 mg/kg. Six patients died with infection although bone marrow had been cleared from leukemic cells. In 15 patients, eradication of leukemic cells could not be obtained. The complete remission rate in adult patients was 43%. Few patients received maintenance therapy with DNR alone, as we were aware of cumulative cardiotoxicity.

Results of special interest were obtained with daunomycin used as a single agent in acute promyelocytic leukemias (5). Before we started to use DNR, complete remission had been obtained in five of 42 patients. A significant improvement was generated by DNR, with 13 of 29 patients (45%) achieving complete remission; however, 16 patients died during the induction phase, mainly from cerebral hemorrhage. A better understanding of the diffuse intravascular clotting underlying this hemorrhagic syndrome prompted the use of systematic prophylactic platelet transfusions associated with heparin therapy (1 mg/kg/day). With this induction regimen 21 of 26 patients (78%) achieved complete remission. The differences between these three remission induction regimens in acute promyelocytic leukemias are significant at the 0.01 level.

Interest in acute promyelocytic leukemias is substantiated by the duration of disease-free interval. Maintenance regimen used the combination of AraC-DNR, AraC-6MP and AraC-Cytoxan; DNR was stopped when a cumulative dose of 20–25 mg/kg was reached. Of all patients, 41% (53% of patients with complete remission) are still in first complete remission. Furthermore, the figure reaches a plateau after 3 years. This curve compared to that observed in other forms of ANLL illustrates the high sensitivity of acute promyelocytic leukemia to DNR alone.

Rubidazone

Experimental data demonstrated that rubidazone at equitoxic doses had an activity similar to that of DNR (Adriamycin [ADR]) against Sarcoma 180, leukemia L1210, mammary carcinomas, and Lewis lung carcinoma (6). Rubidazone (BHDNR), however, appeared three to four times less cardiotoxic than the parent compound (6). These results have prompted phase I and II studies in ANLL. Thirty-seven patients with advanced ANLL, who had received prior DNR, were

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treated with BHDNR alone. Of these 37, 16 (43%) achieved complete remission. No cardiac failure was observed in this small number of patients. Although these patients were probably not resistant to DNR, they had all previously received more than 20 mg/kg of DNR, which prevented its further use (7).

Seventy patients with previously untreated ANLL received BHDNR alone as a remission induction regimen. Of these 70 patients, 40 (57%) achieved complete remission while 15 (21%) died during the induction phase and 15 appeared resistant to BHDNR. The median dose necessary to achieve complete remission was 23 mg/kg (15–32 mg/kg). This dose is exactly twice as high as the dose necessary with DNR. Duration of induced pancytopenia appeared longer than with DNR (mean = 25 days). Of special interest was the fact that complete remission was obtained in 12 of 14 patients with acute monocytic leukemia (M5). Such activity in acute monocytic leukemias led to further study of BHDNR in those cases. Nine of 18 patients with M5 leukemia treated with DNR had achieved complete remission. Treatment with BHDNR alone increased this figure to 76% (37 of 49, $p = .046$). Moreover, duration of complete remission was improved in patients treated with BHDNR, as median duration was 2 months in patients treated with DNR and 9 months in patients treated with BHDNR and receiving CNS prophylaxis.

Therefore, it appears that rubidazone used as a single agent achieves complete remission more readily than DNR. However, as we introduced CNS prophylaxis in the treatment of M5 leukemias together with rubidazone, the effect of rubidazone on duration of remission cannot be estimated.

Detorubicin

Experimental data had shown that the chemotherapeutic index (AD50/LD50) of detorubicin was superior over that of Adriamycin. However, the results were disappointing in ANLL: two of 11 patients with advanced ANLL achieved complete remission with doses averaging 12 mg/kg. The mucosal toxicity appeared to be a dose-limiting factor (8).

Toxicity

These studies with anthracyclines used as single agent in acute leukemias allow for comparison of acute and chronic toxic effects in man. The main acute toxic side effects are bone marrow depression, nausea and vomiting, and alopecia. The incidence of nausea and vomiting (80%) and of alopecia (95%) is the same with ADR, DNR, BHDNR, and detorubicin.

DNR, BHDNR, and detorubicin induce bone marrow depression, although in a different pattern: DNR given as a 2 mg/kg injection daily for 4 or 5 days induces pancytopenia in 94% of patients (9). The nadir of wbc count is reached after a mean interval of 8 days and the duration of pancytopenia averages 15 days. BHDNR induced pancytopenia in 60 of 70 patients (86%) receiving a median dose of 23 mg/kg (7). The nadir of wbc counts was reached after 12 days and the mean duration of pancytopenia was 25 days. Therefore, as compared to DNR, BHDNR is equally as bone marrow-suppressive, but induces a delayed and long lasting pancytopenia. Detorubicin (10 mg/kg) induced pancytopenia in 80% of patients with a mean duration of 12 days, a pattern very similar to the toxicity of ADR.

Mucositis is rarely induced by therapeutic doses of DNR or BHDNR if appropriate local care is performed. On the contrary, mucositis was observed in almost 100% of patients treated with detorubicin when the dose averaged 10 mg/kg; such toxicity prompted the discontinuation of the 5-day schedule.

Myocardial toxicity due to mitochondrial and myofibrillar damage (10) is the major chronic side effect of anthracyclines.

From experimental data previously described (15), detorubicin and DNR appear as cardiotoxic as ADR; rubidazone appears six times less cardiotoxic than ADR. In man, DNR is usually stopped when the cumulative dose reaches 650 mg/sqm, a dose at which the incidence of congestive heart failure is < 5% (11). With rubidazone, the estimated highest non-cardiotoxic dose averages 1800 mg/sqm. We have not seen any cardiac failure in 30 patients receiving from 1300–1800 mg/sqm. Myocardial toxicity in patients treated with rubidazone has been noticed only in patients with previous anthracycline exposure. Therefore, because noninvasive procedures (ECG, echocardiography) do not allow for accurate prediction of cardiotoxicity and as we do not routinely perform myocardial biopsies (13), we use the following rules: (*a*) maximum cumulative dose should not exceed 550 mg/sqm with ADR, 650 mg/sqm with DNR, and 1800 mg/sqm with BHDNR; and (*b*) no anthracycline should be administered in patients with previous angina pectoris or myocardial infarction (12).

Our experience with detorubicin does not allow for accurate determination of cardiotoxicity.

Combination Chemotherapy with Anthracycline in ANLL

Results of two protocols which have been used in our institution since 1976 will be briefly summarized.

14 LAM 76 Protocol

One of the goals of the remission induction regimen was to compare respective values of DNR and BHDNR. All patients received AraC 100 mg/sqm/day for 7 days (continuous infusion). Half of them were randomized to receive BHDNR 12 mg/sqm/day for 3 days, while the other half received DNR 60 mg/sqm/day for 3 days. Courses were repeated if complete remission was not obtained. Maintenance therapy consisted of monthly courses with AraC (200 mg/sqm/day × 5) associated with 6MP or cyclophosphamide or the same anthracycline used in the remission induction regimen. Anthracycline administration was discontinued after 15 months.

Among 195 evaluable patients, complete remission was obtained in 112 (57%), 40 patients (21%) died during the initial phase, and 43 patients (22%) failed to achieve complete remission. The best results were obtained in patients < 15 years with a complete remission rate of 77%, compared to 53% in adult patients. Seventy percent of complete remissions were obtained after the first course of therapy. The results of the remission induction regimens failed to show significant differences between DNR (60% CR) and BHDNR (55% CR). The duration of complete remission was not influenced by the type of anthracycline used: median duration of remission was 10 months with 45% of patients still in first complete remission and the actuarial survival curve reaching a plateau at 24 months.

These results, although comparable to those of the largest series of ANLL, were somewhat disappointing, as neither the complete remission rate nor the duration of remission were improved as compared to previous protocols in ANLL. These results prompted a pilot study using high dose AraC and BHDNR as a remission induction regimen in patients with ANLL treated in protected environment with oral non-absorbable antibiotics (14).

USI 1 Protocol

Induction chemotherapy combined AraC (200 mg/sqm/day) for 5 days given as a continuous infusion and rubidazone (200 mg/sqm/day) for 3 days given as a 45-minute infusion. If leukemic cells had not disappeared from the marrow 10 days after completion of the first course, a second one was initiated with AraC (200 mg/sqm/day) for 3 days and BHDNR (450 mg/sqm) on the first day. The aims of this study were: (a) to achieve a higher remission rate in adult patients; (b) to maximize results of the first course; (c) to study acute toxicity of such a regimen; and (d) to achieve longer remissions, the maintenance regimen being the same as that described for the 14 LAM 76 regimen.

Thirty-seven adult patients with ANLL have been treated using the USI 1 protocol. Median age was 40 years (17–73 years). Patients with acute promyelocytic leukemia were not included in this study. Of these 37 patients, 22 had evidence of infection at presentation, of whom six had major infection. Complete remission was obtained in 27 patients (73%), 19 (51%) after the first course and eight (22%) after a second course. Four patients (13%) survived with leukemic cells in the marrow and six (16%) died during this phase of therapy. Remission rate in patients with acute monocytic or myelomonocytic leukemias (95%) was significantly greater than in the other types. All patients experienced severe pancytopenia. The duration of neutropenia ($<500/\mu l$) averaged 23 days in patients achieving complete remission after the first course and 29 days in patients receiving two courses. While nausea and vomiting were observed in 80% of patients, none experienced mucositis. No cardiac abnormalities were observed. Five patients had transient increase in alkaline phosphatases and transaminases not related to other causes.

The median duration of complete remission was 10.5 months (13 months for patients in CR after one course, 10 months for patients achieving CR after a second course). Forty-five percent of patients achieving complete remission after the first course are still in continuous remission after 24 months, as compared to 12.5% necessitating two courses ($P = 0.05$). These results (15) were further substantiated in an ongoing protocol (16LA79) where this induction regimen achieved 80% complete remission in 39 patients while addition of prednisone-vincristine in one arm and 6-mercaptopurine plus *bis*-methyl-guanyl-hydrazone in another one failed to improve these results.

DISCUSSION

Anthracyclines, when used as a single agent, are the most efficient drugs, achieving complete remission in 40%–50% of patients with untreated ANLL. However, their use is limited by their cardiotoxicity. The therapeutic index of rubidazone in ANLL seems superior to DNR and ADR: the former anthracycline therefore appears the most interesting in the treatment of ANLL, except for the specific interest of daunomycin in acute promyelocytic leukemias. In large series of patients treated with AraC and DNR or BHDNR, we failed to demonstrate significant differences in complete remission rates obtained with those two regimens, the overall complete remission rate being 57%, comparable to the results obtained in other large series of patients treated with conventional reverse isolation (14). Also, the duration of remission was not influenced by the type of anthracycline used. The value of anthracyclines in maintenance therapy of ANLL is still questionable and should be studied specifically. However, it would have been almost impossible to reach doses of DNR ranging from 300 to 500 mg/m², roughly equivalent to the doses of BHDNR that we have used since 1978.

The use of high doses of AraC and rubidazone significantly improved the rate of complete remission in adult patients ($P = .001$) as compared to results of previous regimens. However, as patients were treated in a protected environment with a significant reduction in major infections and deaths (16), the reasons for such a difference could be complex. Preliminary results of a controlled study in a larger series of patients have confirmed this advantage.

These results suggest some prospective goals for the treatment of ANLL with anthracyclines. First, discrimination of patients with "high-risk" ANLL at presentation appears mandatory. Adult patients, in our studies as well as in others (14), share a worse prognosis than children. In adult patients, neither the leucocyte count nor the existence of organomegaly seem of prognostic significance in our experience, the most significant prognostic parameter being the number of courses to complete remission. Classification according to FAB could be of value. Patients with acute monocytic or myelomonocytic leukemias have a better initial prognosis in our pilot study; however, patients with acute monocytic leukemia have a high risk of CNS relapse, which makes CNS prophylaxis mandatory. On the contrary, ANLL patients unclassified using these criteria hardly get into complete remission with the combination of AraC and BHDNR.

The time elapsed between first signs of acute leukemia and diagnosis does not carry any prognostic significance, except in cases classified as refractory anemia with excess of leukemic cells. There are some indications that major cytogenetic abnormalities carry a poor prognosis. Definition of the prognostic value of these points necessitates larger series of patients and is currently under study. However, the significant difference in duration of remission as a function of number of courses necessary to obtain it already allows for discrimination of patients with a very limited chance for long survival: when complete remission has not been achieved after one course of induction regimen, allogeneic bone marrow transplantation or intensification chemotherapy should be considered.

Causes for apparent initial drug resistance must be studied with special reference to pharmacokinetics of drugs and in vitro study of sensitivity of leukemic cells to antitumor agents.

As it appears that duration of remission is significantly longer in patients entering complete remission after the first course of chemotherapy, one of our goals is to achieve higher remission rate with one course of chemotherapy. As previously stated, the addition of other drugs failed to improve these results. On the other hand, use of still higher doses of AraC and BHDNR has achieved 83% complete remissions in a pilot study (in preparation).

Duration of remission does not seem to be significantly modified with either intermittent or continuous maintenance therapy. The value and toxicity of late intensification are still under study. Other procedures such as auto-transplantation with bone marrow obtained in complete remission and immunologically deprived from leukemic cells are still experimental and have been studied in animal systems.

Finally, it appears necessary to look for active agents with an increased therapeutic index; detorubicin failed to show improvement over daunomycin and rubidazone in our studies. Whether these new drugs will belong to the anthracycline group or to another series is questionable: among anthracycline derivatives, aclacinomycin A warrants further study (17).

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Studies With Anthracyclines in Pediatric Acute Nonlymphocytic Leukemia[1]

Ronald L. Chard, Jr.[2,*]

SUMMARY

The anthracyclines, daunomycin and Adriamycin, have become two of the most important chemotherapeutic agents in the treatment of pedriatric acute nonlymphocytic leukemia. Data are presented on 306 children treated after initial diagnosis with an anthracycline or one of the chemotherapeutic agents. The remission rate was 65%. When not used during maintenance therapy, the anthracyclines have been shown to be effective in inducing a second remission even though the drug was used during initial therapy. An ongoing randomized study utilizing Adriamycin in induction of remission and during maintenance therapy is also discussed.

[Cancer Treat Rep 65 (Suppl 4):77–81, 1981]

Daunomycin and Adriamycin have been shown to be very effective in the treatment of pediatric acute nonlymphocytic leukemia. Initial studies in 1967 showed daunomycin was an effective agent in refractory acute leukemia of childhood (1), an observation confirmed by others (2,3) and shown to hold true also for Adriamycin (4).

In 1974 the Childrens Cancer Study Group began a study utilizing daunomycin and 5-azacytidine for reinduction of children with acute nonlymphocytic leukemia who had relapsed while receiving cytoxan, cytosine arabinoside, 6-thioguanine, vincristine, and prednisone. All bone marrows on these children were reviewed by a single pathologist for accuracy of diagnosis, both on their original diagnostic bone marrow and at the time of relapse.

The induction schema is shown in figure 1; daunomycin in 30 mg/m^2 iv pulse Days 1, 2 and 3, and 5-azacytidine 100 mg/m^2 iv pulse Days 1, 2, 3, 4 and 5. The course was repeated on Day 14 and a single consolidation course given on Day 28. Remission was maintained with 5-azacytidine alone.

The results are shown in table 1. Thirty-four patients were entered and evaluable. Fourteen patients achieved a complete remission (41%), four a good partial remission (5%–15% blasts in the marrow), for a combined complete and good partial remission rate of 52%. Sixteen patients had no response and five patients developed CNS involvement while on the study. Remission duration was short, with a median of 190 days and a range of 26–488 days. For the 16 non-responding patients the median duration on study was only 61 days. Toxicity, shown in table 2, was hematologic and gastrointestinal. There was no cardiotoxicity, but this would be anticipated as the highest cumulative dose of daunomycin for any patient was 270 mg/m^2 (5).

Because of the success of this induction combination, a study (CCG-241) was started in June 1975 utilizing daunomycin and 5-azacytidine with other agents in children with newly diagnosed acute nonlymphocytic leukemia. Again, all slides were reviewed by a single pathologist for confirmation of the diagnosis. The treatment schema is shown in figure 2. Daunomycin was given 30 mg/m^2 iv pulse daily for 3 days, 5-azacytidine 50 mg/m^2 iv pulse bid for 4 days, cytosine arabinoside 25 mg/m^2 iv pulse tid for 4 days, prednisone 40 mg/m^2/day po for 4 days, and vincristine 1.5 mg/m^2 iv on Day 1 only. The course was repeated every 14 days for a minimum of four courses before beginning maintenance therapy.

One hundred sixty-three patients were eligible for evaluation. Induction results, shown in table 3, are broken down by prognostic factors. Sex and age appeared to be the only significant factors affecting induction results with this combination chemotherapy. Induction of remission varied from 61.9% for children greater than 10 years of age to 89.2% for children 5–10 years of age (6). Remission duration was disappointing

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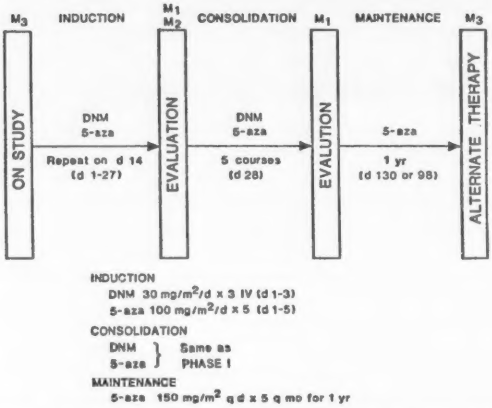
M3
INDUCTION
M1
M2
CONSOLIDATION
M1
MAINTENANCE
M3
ON STUDY
DNM
5-aza
Repeat on d 14
(d 1-27)
EVALUATION
DNM
5-aza
5 courses
(d 28)
EVALUTION
5-aza
1 yr
(d 130 or 98)
ALTERNATE THERAPY
INDUCTION
DNM 30 mg/m2/d x 3 IV (d 1-3)
5-aza 100 mg/m2/d x 5 (d 1-5)
CONSOLIDATION
DNM
5-aza
Same as
PHASE I
MAINTENANCE
5-aza 150 mg/m2 q d x 5 q mo for 1 yr

FIGURE 1.—Childrens Cancer Study Group (CCG-242). Reinduction of patients with ANLL.

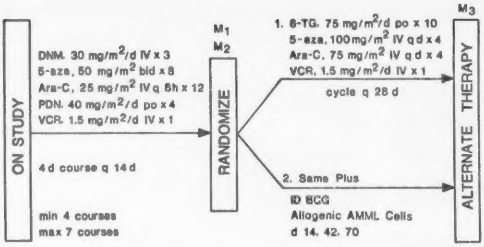
ON STUDY
DNM. 30 mg/m2/d IV x 3
5-aza, 50 mg/m2 bid x 8
Ara-C, 25 mg/m2 IV q 8h x 12
PDN. 40 mg/m2/d po x 4
VCR. 1.5 mg/m2/d IV x 1
4 d course q 14 d
min 4 courses
max 7 courses
M1
M2
RANDOMIZE
1. 6-TG. 75 mg/m2/d po x 10
5-aza, 100 mg/m2 IV q d x 4
Ara-C, 75 mg/m2 IV q d x 4
VCR, 1.5 mg/m2/d IV x 1
cycle q 28 d
2. Same Plus
ID BCG
Allogenic AMML Cells
d 14, 42, 70
M3
ALTERNATE THERAPY

FIGURE 2.—Childrens Cancer Study Group (CCG-241). Induction therapy for patients with ANLL.

and is shown in figure 3. There was no difference between the chemotherapy arm only and the chemotherapy-immunotherapy arm.

Hematologic toxicity was very frequent with 115 of 163 (71%) of patients experiencing moderate to severe marrow suppression. Thirteen patients developed moderately severe gastrointestinal toxicity, two developed severe liver toxicity, and one developed neurologic impairment.

There were 32 deaths during induction; the causes were sepsis in 12 patients, pneumonia in three, intracranial hemorrhage in seven, cerebral vascular accident in one, disseminated intravascular coagulation in three, cardiac arrest in two, progressive disease or otherwise unexplained in two, probable infection with pancytopenia in one, and gastrointestinal hemorrhage in one. The cardiac deaths could not be attributed to daunomycin cardiotoxicity.

Because the maintenance was disappointing and not as long as shown in a previous CCSG study, the study was modified in February 1978. Induction remission was the same as before, but as shown in figure 4 (CCG-241A), maintenance was changed as follows. Immunotherapy was discontinued and chemotherapy became continuous. From February 1978 until February 1979, 120 eligible patients were entered on this study. Ninety-five were evaluable and 62 (65%) achieved a complete remission. Toxicity remained primarily hematologic with infection a major problem.

TABLE 1.—Childrens Cancer Study Group (CCG-242). Reinduction of patients with ANLL

Patients		Response			
Entered	Evaluable	M1	M2A	M3	CNS
34	34	14	4	16	5

TABLE 2.—Childrens Cancer Study Group (CCG-242). Reinduction of patients with ANLL

	Induction						Maintenance					
Toxicity Grade	0	1	2	3	4	Total	0	1	2	3	4	Total
Hema	10	2	4	16	0	32	6	6	4	2	0	18
GI	17	10	3	2	0	32	8	8	2	0	0	18
Cardiac	32	0	0	0	0	32	18	0	0	0	0	18
Liver	32	0	0	0	0	32	18	0	0	0	0	18

CCSG drug toxicity criteria

Toxicity	Drug
0 None	
1 Mild	Continue
2 Moderate	Reduce
3 Severe	Stop
4 Life-threatening	

Table 3.—Childrens Cancer Study Group (CCG-241). Induction remission rate by prognostic factors

	Groups	Rate	M1		M2A	Total
Sex	Males	64.3%	42		12	84
	Females	79.7%	49		14	79 p=.04
Race	Non-white	82.1%	18		5	28
	White	69.6%	73		21	135 N.S.*, p=.28
Age	<5 yrs.	74.0%	28		9	50
	5–10 yrs.	89.2%	25		8	37} N.S., p=.14
	10 yrs.	61.9%	39		9	76} p=.01
WBC	<20 × 10^9/L	75.8%		72		95
	>20 × 10^9/L	66.2%		45		68 N.S., p=.24
Leukemic type	AML	73.1%		57		78
	AMML	62.6%		10		16 N.S., p=.59

*NS = nonsignificant

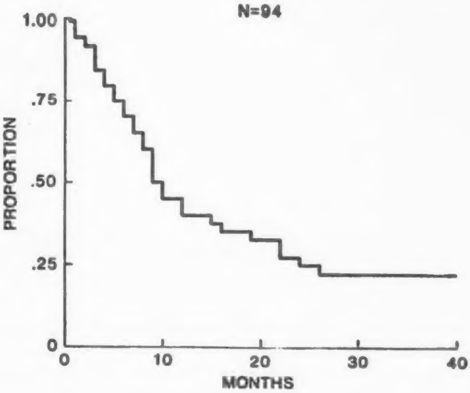

Figure 3.—Childrens Cancer Study Group (CCG-241). Marrow relapse from start of maintenance for all randomized patients with ANLL.

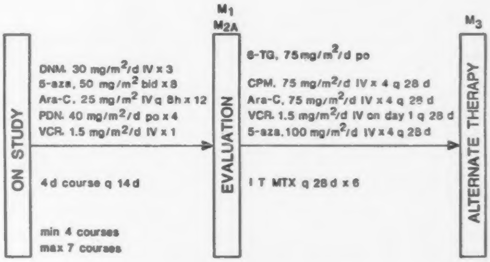
ON STUDY
DNM. 30 mg/m2/d IV x 3
5-aza, 50 mg/m2 bid x 8
Ara-C, 25 mg/m2 IV q 8h x 12
PDN. 40 mg/m2/d po x 4
VCR. 1.5 mg/m2/d IV x 1
4 d course q 14 d
min 4 courses
max 7 courses
M1
M2A
EVALUATION
6-TG, 75 mg/m2/d po
CPM. 75 mg/m2/d IV x 4 q 28 d
Ara-C, 75 mg/m2/d IV x 4 q 28 d
VCR. 1.5 mg/m2/d IV on day 1 q 28 d
5-aza. 100 mg/m2/d IV x 4 q 28 d
I T MTX q 28 d x 6
M3
ALTERNATE THERAPY

Figure 4.—Childrens Cancer Study Group (CCG-241A). Induction therapy for patients with ANLL.

In this study there were 22 deaths during induction, one prior to treatment, three due to hemorrhage, two respiratory arrest, three intracranial hemorrhage, four cardiac arrest (Days 17, 24, 25 and 103), upper G.I. bleeding in one, sepsis in five, unknown in two, and daunomycin cardiomyopathy versus CNS hemorrhage in one patient (7). Again, the cardiac deaths could not be attributed to daunomycin cardiotoxicity.

Children with acute nonlymphocytic leukemia who initially achieve a remission with daunomycin, but do not receive it during maintenance therapy, can be successfully reinduced into a second remission. In February 1977, a study was opened for patients relapsing on CCSG-241 and CCSG-241A. The treatment schema is shown in figure 5 (CCSG-243). On relapse patients were randomly reinduced with daunomycin 30 mg/m²/day iv

Table 4.—Childrens Cancer Study Group (CCG-243). Reinduction of patients with ANLL

	Patients							Response			
	Ent.	Elig.	On study	Off study	Eval tox.	IT	Eval.	M1	M2A	M2B	M3
Reg. I	36	36	9	27	28	3	28	19	4	1	4
Reg. II	34	33	7	26	23	5	23	13	5	1	4
Total	70	69	16	53	51	8	51	32	9	2	8

TABLE 5.—Childrens Cancer Study Group (CCG-243). Reinduction of patients with ANLL

Induction		Regimen I						Regimen II					
Toxicity	Grade	0	1	2	3	4	Total	0	1	2	3	4	Total
Hema		2	0	2	16	8	28	0	0	2	17	4	23
GI		19	3	4	2	0	28	14	7	1	1	0	23
Cardiac		27	0	0	1	0	28	23	0	0	0	0	23

CCSG drug toxicity criteria

Toxicity	Drug
0 None	
1 Mild	Continue
2 Moderate	Reduce
3 Severe	Stop
4 Life-threatening	

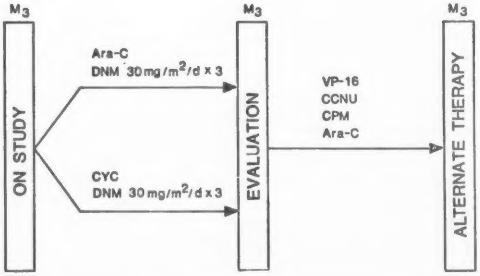

FIGURE 5.—Childrens Cancer Study Group (CCG-243). Reinduction of patients with ANLL.

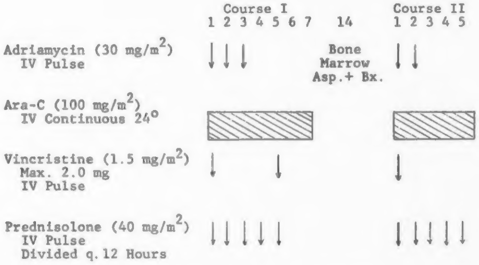

FIGURE 6.—Sidney Farber Cancer Center. Induction schema for patients with ANLL.

pulse × 3 days and cytosine arabinoside 100 mg/m²/day iv pulse bid × 7 days, or cyclocytidine 600 mg/m²/day subcutaneously × 7 days. Maintenance is as shown.

Induction results are shown in table 4. There are 28 evaluable patients on regimen 1 (cytosine arabinoside) and 19 obtained a complete remission (68%). Twenty-three patients are evaluable on regimen 2 (cyclocytidine) and 13 obtained a complete remission (57%) (8). Patients who are off study are those who failed to achieve remission, relapsed after achieving remission, or died while in remission. On study patients are those alive and still in induction at the time of this report. As shown in table 5, only one patient of 50 so treated has shown any cardiotoxicity. At the time of this report it has not been fatal, but daunomycin was discontinued on this patient. Of the 60 eligible patients entered, two died too early to evaluate induction drug toxicity. The remaining 16 patients are too early in their induction program to evaluate drug toxicity.

The Sidney Farber Cancer Center in Boston has used an equally impressive induction program for induction of remission in acute nonlymphocytic leukemia in children and adults. This is shown in figure 6. Adriamycin 30 mg/m² by iv pulse is given daily for 3 days with cytosine arabinoside 100 mg/m² by continuous iv infusion for 7 days, vincristine 1.5 mg/m² by iv pulse on Days 1 and 5, and prednisolone by iv infusion 40 mg/m² in two divided doses for 5 days. If complete remission is not achieved after the first course, a second modified course is given as shown. Results are shown in table 6. Thirty-five of 48 children from 0 to 17 years of age achieved a complete remission (73%). Of great interest is the rapidity of achieving the remission with this combination. Twenty-six (54%) achieved complete remission after one course.[3]

In summary, the anthracycline drugs, daunomycin and Adriamycin, have been shown to be two of the most effective agents in the treatment of children with acute nonlymphocytic leukemia. Data is presented showing that since 1974 306 children with acute nonlymphocytic leukemia have received either daunomycin or Adriamycin as part of their combination chemotherapy with 198 (65%) achieving a complete remission.

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[3] Weinstein HJ. Personal communication, 1979.

TABLE 6.—Remission induction (10-1-79)

	Age 0–50	Age 0–17	Age 18–50
# Patients	83	48	35
# Complete remission	58 (70%)	35 (73%)	23 (66%)
# Courses to CR			
1	40	26	14
2	18	9	9

The Childrens Cancer Study Group has just started a new study for pediatric acute nonlymphocytic leukemia. Induction therapy for all patients is Adriamycin 30 mg/m² iv pulse × 3 days and cytosine arabinoside 100 mg/m² per day by continuous infusion for 7 days. A second induction course of Adriamycin 30 mg/m² iv pulse × 2 days and cytosine arabinoside 100 mg/m² iv by continuous infusion for 5 days will be given at Day 14.

All children who have an HLA, MLC compatible sibling marrow donor will then be eligible for bone marrow transplantation in first remission. All children not eligible for bone marrow transplantation will be randomly assigned to two chemotherapy arms. Arm 1, continuous chemotherapy, will utilize daily 6-thioguanine and monthly pulses of vincristine, cytosine arabinoside, 5-azacytidine, and cyclophosphamide. Arm 2, intermittent sequential chemotherapy, will utilize Adriamycin and cytosine arabinoside (course A), prednisolone, vincristine, methotrexate and 6-mercaptopurine (course B), 5-azacytidine and Adriamycin (course C), BCNU and cyclophosphamide (course D), and 6-thioguanine and cytosine arabinoside (course E). Adriamycin will be discontinued when a total of 480 mg/m² has been reached. All patients will receive CNS prophylaxis.

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Prognostic Factors in Response to Anthracyclines[1]

Kenneth B. McCredie,* Michael J. Keating, Gerald P. Bodey, Edmund A. Gehan, Terry Smith, and Emil J. Freireich[2]

SUMMARY

Effective chemotherapy using anthracycline antibiotics and cytosine arabinoside has substantially improved the response in patients with acute myeloblastic leukemia. Prognostic factors have now been developed that can predict not only for response but for the duration of response. In the future, it may be possible to individualize chemotherapy.

[Cancer Treat Rep 65 (Suppl 4):83-86, 1981]

Effective chemotherapy for the management of adult acute leukemia has been available for slightly more than 10 years. The first drug to demonstrate substantial activity as a single agent in the management of this disorder was cytosine arabinoside (1). The subsequent anthracycline antibiotics, particularly daunorubicin and Adriamycin (doxorubicin), have shown substantial response rates in adult acute leukemia. The combination of these agents with cytosine arabinoside plus vincristine, prednisone, or 6-thioguanine has become the mainstay in the majority of chemotherapeutic programs (2,3). In addition to improved response rates, there also appears to be an increased duration of complete remission, with a number of institutions now reporting long-term survivors and potential cures in these groups of patients (4-6).

In addition to the improvement in drug therapy, substantial improvements have been made in the supportive care of patients during myelosuppressive phases of remission induction therapy. The introduction of new antibiotics, particularly the semisynthetic penicillins and the aminoglycosides, has substantially improved treatment for gram-negative sepsis, improved hematological support with both platelet and granulocyte transfusions, and reduced the morbidity and mortality associated with thrombocytopenia and granulocytopenia.

To assess what factors would predict for response, a stepwise regression analysis was performed from pretreatment data collected from 325 patients who were subsequently treated at M. D. Anderson Hospital with anthracycline antibiotic combinations and cytosine arabinoside. A number of pretreatment characteristics were individually and separately assessed as being predictive of complete remission (table 1). The total white cell count at diagnosis was considered in the analysis as were other factors and were not found to be as important as the seven listed.

The presence of a preceding hematologic disorder for for longer than 1 month prior to the diagnosis of acute leukemia imparted a poorer prognosis, with a response rate of 29% compared to a 69% response rate for those patients who did not have a preleukemic disorder. Similarly, for patients older than 50 years, the response rate was 46% compared to a response rate of 76% for patients under the age of 50. In those patients in whom insufficient numbers of metaphases could be analyzed on chromosome preparations, the response rate was 32%, compared to 67% in those in whom chromosome preparations could be analyzed. Patients

TABLE 1.—Pretreatment characteristics in 325 patients predicting for response

1. Preleukemia
2. Age
3. Insufficient metaphases
4. Temperature
5. Serum creatinine
6. Auer rods
7. Hepatomegaly

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with a temperature greater than 101°F on admission had a response rate of 43% compared to 71% for those who were not febrile. With elevated serum creatinine and the presence of established renal failure, the response rate was 45% compared to 65%. The presence of Auer rods was a favorable prognostic sign, with 68% responding compared to 55% of those patients in whom Auer rods could not be demonstrated. Hepatomegaly at the initial physical examination imparted a poorer prognosis; only 30% of the patients with this condition responded.

The regression equation for calculating the probability of response was developed on these pretreatment characteristics (fig 1). Table 2 shows the observed response rates predicted on the basis of this regression model in the 325 patients from whom it was derived. To assess whether this regression equation could accurately predict for response, it was utilized to predict the probability of response prospectively in 107 patients who received a similar combination of anthracyclines and cytosine arabinoside with vincristine and prednisone. The result of this prospective analysis is shown in table 3 and shows a close correlation of the observed and predicted probability for response.

TABLE 2.—Observed response rates by predictions based on regression model — 325 patients

Predicted prob. of CR	Total no. observed	No. responding (%)
< .20	36	4 (11)
.20–	38	12 (32)
.40–	55	26 (47)
.60–	74	54 (73)
.80–	97	85 (88)

In addition to predicting probability for response, a further set of characteristics was identified, using a similar regression analysis as being responsible for predicting the duration of response in those patients achieving a complete remission. These factors are listed in table 4. A low lactate dehydrogenase (LDH), normal fibrinogen level, a single course of chemotherapy before complete remission, the diagnosis of acute

$$\ln\left(\frac{p}{1-p}\right) = .490 - 1.688\,(\text{pre-leuk.} - .19) - .033\,(\text{age} - 45.87)$$
$$+\ 1.486\,(\text{insuf. met.} - 1.85) - 1.141\,(\text{temp.} - 1.34) - .805$$
$$-\ .570\,(\text{dx. Auer} - .47) - 1.416\,(\text{liver} - .05)$$
$$\text{where } p = \text{probability of CR}$$

FIGURE 1.—Regression equation for calculating probability of response.

TABLE 3.—Comparison of observed and predicted probabilities of response
A prospective analysis of 107 patients*

Predicted probability of response	Total no. observed	Observed no. (PC) responding	Expected no. responding
0 - .19	15	2 (13)	1.832
.20 - .39	15	5 (33)	4.398
.40 - .59	18	7 (39)	9.089
.60 - .79	27	22 (81)	18.822
.80 - 1.00	32	28 (88)	28.696

*Chi-square statistic is 1.131 for 4 degrees of freedom, $P = .889$.

TABLE 4.—Summary of regression analysis relating length of response to patient characteristics

Characteristic	*P*	Log-likelihood	Favorable	Relative risk Unfavorable	Ratio
LDH	< .001	−437.506	.66	1.31	1.98
Fibrinogen	.001	−432.464	.71	1.52	2.14
Course to CR	.004	−428.426	.79	1.80	2.28
AML	.081	−426.909	.83	1.54	1.86
Time to halving leukemic cells	.031	−424.570	.66	1.42	2.15
Age	.056	−422.749	.93	2.10	2.26

myeloblastic leukemia, a time of less than 10 days to the halving of the total number of leukemic blast cells in the peripheral blood, and an age less than 50 years imparted a good prognosis and were associated with a prolonged complete remission.

The survival from the start of chemotherapy for 325 patients is shown in figure 2, and the duration of complete remission is shown in figure 3. The survival curve in the initial 325 patients treated with anthracyclines and cytosine arabinoside is a triphasic one with the vertical lines representing those patients who are still alive and could still affect the curve. The initial steep part of the response curve represents those patients who failed to achieve a complete remission; the subsequent less steep part of the curve represents those patients achieving a complete remission who subsequently relapsed. The curve tends to become horizontal at approximately 24 months, with between 10% and 20% of all patients remaining in long-term complete remission. The initial constant relapse rate and the horizontal portion of the curve representing those patients who are potential long-term survivors is demonstrated again in the curve representing patients achieving complete remission (fig 4).

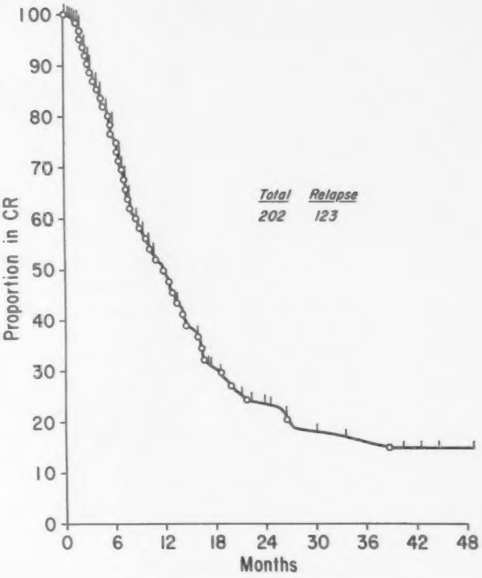

FIGURE 3.—Duration of CR, all patients.

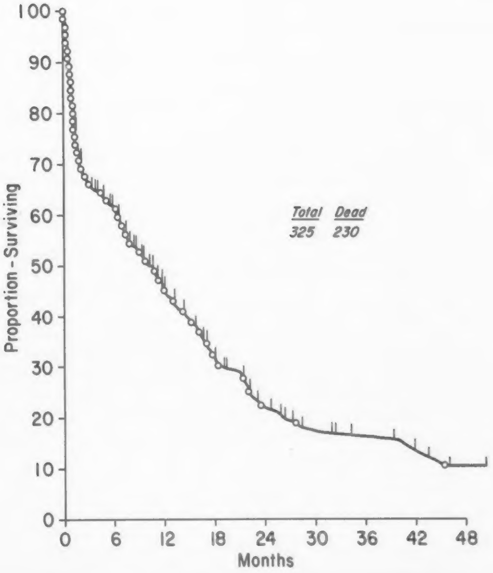

FIGURE 2.—Survival from start of chemotherapy, all patients.

In addition to the patients currently being treated with anthracycline plus cytosine arabinoside, we have retrospectively analyzed 288 previously untreated patients with acute myeloblastic leukemia who subsequently received remission induction therapy at M. D. Anderson Hospital between 1965 and 1973. Of the initial 288 patients, 115 achieved a complete remission and 23 patients have survived for more than 5 years from the beginning of chemotherapy. Two of the 23 patients have subsequently died, one of an unrelated cause, one following relapse. Twenty patients remain in continuous complete remission for a period of up to 9 years. Of the long-term survivors, all received cytosine arabinoside during induction and/or maintenance therapy. The majority of patients achieving long-term complete remission were in a group of patients that received cytosine arabinoside with or without anthracycline antibiotics between 1971 and 1973. Seventeen of 69 patients in this group (24.6%) are long-term survivors, 70 have survived for longer than 5 years, and all 70 have been off chemotherapy for periods of 3 years or longer.

When the factors that predicted for long-term complete remission were analyzed, they were found to include treatment with cytosine arabinoside and the anthracycline antibiotics, a low serum LDH, a high blast-to-mature myeloid ratio in the bone marrow, a diagnosis of acute promyelocytic leukemia, the presence

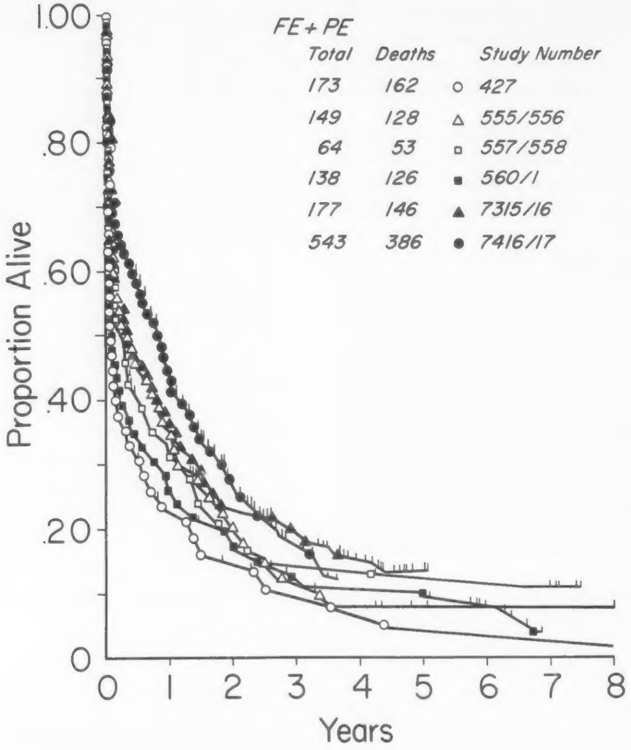

FIGURE 4.—Survival of acute leukemia patients from first treatment.

of greater than 4% eosinophils in the bone marrow, and a rapid decrease in bone marrow infiltrate. These factors also had a favorable effect on the long-term survival of these patients.

Utilizing the data accumulated on remission induction, duration of complete remission, and long-term survival, it is now probable that one can predict which patients have favorable prognosis and what the probability of long-term survival and potential cure are. In addition, it will be possible to predict which patients have poorer prognosis for both achieving complete remission and long-term survival. With this data available, it may be possible to individualize the treatment for a patient with a prognosis in the hopes of improving his response and duration of survival.

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Progress in Acute Myelogenous Leukemia[1]

Robert Peter Gale[2,3,*]

SUMMARY

There has been substantial recent progress in the treatment of acute myelogenous leukemia (AML). Intensive induction chemotherapy with cytosine arabinoside and daunorubicin with or without 6-thioguanine will induce remission in over 70% of patients, with median remission of 1–2 years in most series. Attempts to prolong remissions with maintenance chemotherapy, immunotherapy, late intensification or central nervous system prophylaxis have been disappointing and there are no convincing data that these modalities are useful. Nevertheless, an increasing proportion of patients with AML have remissions of 3 or more years, and a small proportion may be cured.

[Cancer Treat Rep 65 (Suppl 4):87–92, 1981]

Over the past 20 years there has been substantial progress in the treatment of acute myelogenous leukemia (AML). Most notable have been the development of new and effective anti-leukemic drugs such as cytosine arabinoside (ara-C) and daunorubicin and advances in supportive care such as broad-spectrum antibiotics and platelet transfusions. Despite these recent advances, many important questions remain regarding the optimal therapy of AML. I will briefly review current concepts of the treatment of AML stressing concepts of therapy and highlighting unresolved issues. At times I have been forced to choose between seemingly contradictory data or to base opinions on unconfirmed reports. I hope that some of these unresolved issues will be answered in controlled trials in the near future.

Since AML was first described in the 19th century, the diagnosis has portended a fatal outcome. Survival of untreated patients was brief, typically less than 6 months. The 1940s and 1950s witnessed the introduction of the folic acid antagonists, the first effective drugs for the treatment of acute leukemia. Subsequently, numerous drugs have been discovered or synthesized that have been reported to be active in patients with AML. Notable among these are the purine analogs 6-mercaptopurine and 6-thioguanine, the pyrimidine analog ara-C, and the anthracycline antibiotic, daunorubicin. Modern therapy of AML has evolved from a careful sequential evaluation of these drugs used either singly or in combination. Some of these data have been reviewed by others in this volume and elsewhere (1–3). I shall focus my attention on a brief review of the sequential studies of single- and multiple-drug therapy of AML.

The treatment of AML can be divided in operational phases indicated in figure 1. These include *induction*, during which high doses of drugs are given to achieve a remission; *consolidation*, during which high doses of drugs are given to eliminate clinically undetectable leukemic cells; *CNS therapy*, during which radiation or intrathecal chemotherapy may be used to eradicate leukemia cells in the CNS that may have escaped systemic chemotherapy; *maintenance*, during which lower doses of drugs are given to prevent leukemic recurrence; and *intensification*, during which high doses of drugs are given to patients in remission for several months to years. I shall review each of the treatment phases separately.

Results of single- and multiple-drug *induction* therapy in AML are summarized in figure 2. 6-Mercaptopurine and 6-thioguanine are modestly effective anti-leukemic drugs inducing remissions in 10%–15% of patients with AML (4–6). Ara-C is considerably more effective as a single agent, with a remission induction rate of 25%

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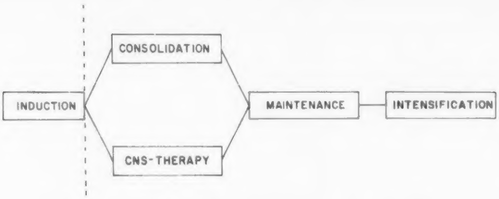

FIGURE 1.—Operational phases of treatment of AML.

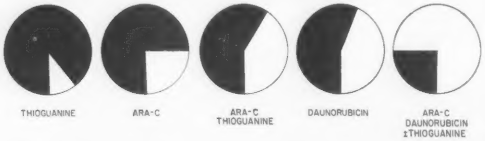

FIGURE 2.—Results of single- and multiple-drug therapy in AML. Remission induction rate is shown by the white portion of the circle.

when given at optimal dose and schedule (7–9). The combination of ara-C and 6-thioguanine induces remissions in 30%–40% of patients with AML, results superior to those achieved with either drug alone (10–13). Daunorubicin, the most effective single drug in AML, produces remissions in 40%–50% of patients at optimal dose and schedule (14–16). Three daily doses of daunorubicin (60 mg/m²/day) added to a 7-day schedule of ara-C (100–200 mg/m²/day) with or without thioguanine produces remissions in 70%–80% of patients with AML (17–22). Several features of these intensive 7-day induction regimens combining daunorubicin and ara-C are noteworthy. First, marrow aplasia is rapidly achieved with intensive therapy, median time to complete remission is 30 to 50 days in several series. This rapid induction rate results in a period at risk for infections and hemorrhage that is substantially shorter than that associated with less intensive regimens. Nevertheless, most patients require systemic antibiotics and blood transfusions; oral non-absorbable antibiotics, granulocyte transfusions, and protected environments are also used at many centers. Two thirds of patients who eventually respond to induction chemotherapy achieve a remission following a single induction course. The remaining patients require two or rarely three courses. The rapidity of induction may be important in older patients who tolerate marrow aplasia poorly. For example, several series have reported response rates exceeding 70% in patients over 60 years of age (20–22). On the average, however, older patients probably respond less well than do young patients. It is also noteworthy that comparably good response rates are seen in all morphologic subtypes of AML. Recent data, discussed in this volume, suggest that patients with progranulocytic leukemia may respond as well to daunorubicin alone as to the combination of daunorubicin and ara-C (16).

There are several unresolved questions regarding induction chemotherapy in AML. The first involves optimal drug doses and schedules. Seven-day induction cycles are probably superior to 5-day cycles although some investigators have reported good results with the latter (23). Ten-day induction cycles do not produce more remissions and toxicity is probably increased (24). There are also no convincing data that 10-day induction regimens produce longer remissions. The question of whether daunorubicin or doxorubicin is superior for induction chemotherapy in AML is unresolved. Few controlled trials have been reported, but most data suggest an advantage for daunorubicin, primarily because it is more myelosuppressive than doxorubicin (25). Data that daunorubicin is less cardiotoxic than doxorubicin at doses producing comparable myelosuppression is reviewed in this volume and elsewhere (26). The question of whether the daunorubicin should be given on the first three or last three days of the 7-day induction cycle is unresolved, and there are no convincing data that either schedule is superior (14). A more controversial issue is the optimal dose and schedule of ara-C administration. Common schedules are 100 mg/m²/day continuous iv infusion or 100 mg/m² rapid iv infusion every 12 hours. These schedules probably produce comparable results. A dose of ara-C of 200 mg/m²/day continuous iv infusion produces severe gastrointestinal toxicity, and there is no evidence of any substantial benefit in terms of more rapid remissions or higher remission rates. Finally, it is unresolved whether 6-thioguanine is an important component of these intensive regimens. We have included it in the TAD regimen based on data indicating a synergistic effect between ara-C and 6-thioguanine in less intensive 5-day induction regimens.

Granulocyte transfusions and laminar air flow (LAF) environments are not necessary for successful induction therapy of AML but are used at many centers. *Therapeutic* granulocyte transfusions may be useful in patients with documented infections, particularly bacteremia (27,28). *Prophylactic* granulocyte transfusions are probably not useful in patients with AML who receive induction chemotherapy (29). Protected environments, LAF rooms, and oral non-absorbable antibiotics may also help to decrease the incidence of infections.

Once remission is achieved, the next objective is to prevent leukemic recurrence using *consolidation* therapy, *maintenance* therapy, or both. Consolidation therapy involves one to three cycles utilizing drug and dose schedules similar to those used for remission induction and is typically given as soon as remission is achieved. Maintenance therapy utilizes multiple cycles of lower doses of the same or new drugs. It is typically given following consolidation therapy and may be continued for several years. I will combine my analysis of consolidation and maintenance therapy since they have not been separately evaluated in controlled clinical trials.

A typical remission duration curve of intensively-treated patients with AML who receive consolidation and maintenance chemotherapy is illustrated in figure 3. Median remission durations range from 12 to 24 months in most series. In several large studies 10%–30% of patients may remain in remission for periods exceeding 3 years. Factors such as age, morphologic type, and the number of cycles required to achieve remission do not appear to influence length of remission. Studies indicating that factors such as the drugs used for induction chemotherapy, the rate of response, LDH, percent myeloblasts at time of diagnosis, eosinophilia, and SGOT during the consolidation phase may influence remission duration are reviewed elsewhere in this volume. Most of these data are controversial and of limited critical import.

Critical evidence that consolidation or maintenance therapy prolong remission is lacking, and only small differences in remission duration have been reported in two published randomized trials (30,31). It is important to remember that these patients did not receive modern intensive induction regimens and that the drugs, doses, and schedules of their maintenance chemotherapy may not have been optimal. Several observations regarding maintenance therapy require comment. One is the relative constancy of remission duration regardless of whether simple, non-intensive therapy or complex intensive therapy is given. A second observation is that median remission duration has probably not improved substantially in recent years. One possible explanation of these findings is that modern induction regimens achieve optimal leukemia cell-kill and that additional therapy, such as maintenance chemotherapy, has little effect. A second consideration is that the median is a poor measure of remission duration in a disease with such a poor prognosis and that greater weight should be placed on the proportion of patients remaining in remission at 2 or more years. For example, patients with a poor prognosis who might previously have failed less intensive induction therapy may now achieve brief remissions that shorten median remission duration. This effect could obscure a beneficial effect of maintenance therapy on remission duration in good-risk patients.

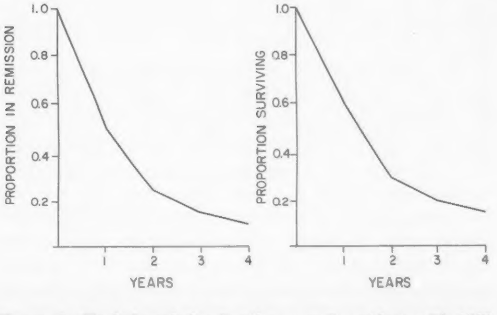

Figure 3.—Typical remission duration curve for patients with AML who receive intensive consolidation and maintenance chemotherapy.

Whether consolidation and/or maintenance therapy should be routinely given cannot be critically answered with the currently available data. In a recently completed study at UCLA, patients received two courses of intensive consolidation chemotherapy; they did not receive maintenance chemotherapy (fig 4). Preliminary analysis of these data indicates a median remission duration comparable to our previous studies in which patients received maintenance chemotherapy.

There are numerous and contradictory reports concerning the role of immunotherapy in AML. Several early studies reported that immunotherapy prolonged remission duration (32–36). With few exceptions these early differences have disappeared or have not been confirmed in randomized trials (37,38). Some investigators have suggested that immunotherapy improves survival by increasing the frequency of second remissions or that immunotherapy alone produces remissions comparable to maintenance chemotherapy and is less toxic. This may be because neither therapy is particularly effective. One possible explanation for the disappointing results achieved with immunotherapy may be that it is usually given when patients are also receiving maintenance chemotherapy, the immunosuppressive effects of which may negate any possible beneficial effect of immune stimulation.

Questions regarding the potential importance of CNS therapy in the treatment of AML have been prompted by two observations: an increasing incidence of meningeal leukemia in patients with AML (39–41),

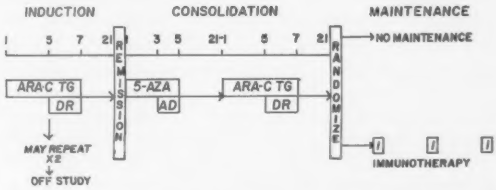

Figure 4.—Design of current trials in which patients with AML receive intensive induction and consolidation chemotherapy and are then randomized to receive or not to receive intralymphatic and systemic immunotherapy.

and the demonstration of the important role of CNS therapy in prolonging remissions in acute lymphocytic leukemia. Several non-randomized and randomized trials have studied the role of CNS therapy. In general they have shown that cranial radiation or intrathecal chemotherapy with ara-C or methotrexate can prevent CNS leukemia but that this does not prolong the duration of systemic remissions. Because of the relatively low incidence of meningeal leukemia, 5%-10% in most studies, CNS prophylaxis should not be routinely given to patients with AML. If meningeal leukemia develops, it can usually be effectively treated. Patients with AML who develop neurologic or psychiatric symptoms should have appropriate studies to determine if they have meningeal leukemia. Some centers recommend routine cerebrospinal fluid examinations in patients with AML in remission for 2 or more years.

Finally, I would like to consider the role of *intensification* therapy in AML. Intensification can be defined as high-dose chemotherapy given to patients in remission for 6 or more months. Patients may receive new drugs or those used for remission induction. Initial enthusiasm for intensification therapy followed reports of uncontrolled trials at M. D. Anderson Hospital that indicated a favorable effect on remission duration (42,43). Subsequent data have been less impressive and there are no randomized trials addressing this important question. Such studies are clearly needed before intensification can be considered to be effective in AML or can be routinely recommended. A recent report indicated that routine maintenance chemotherapy may produce comparable results.

One innovative approach to the treatment of AML is bone marrow transplantation. The basic concept is to give high doses of drugs and radiation followed by rescue with bone marrow from an HLA-identical sibling (44,45). This approach might be effective for two reasons. First, one can give higher doses of drugs and radiation than could be given without marrow rescue. In this regard, marrow transplantation can be considered as a very intensive form of consolidation or intensification therapy. A second potential benefit of marrow transplantation is an anti-leukemic effect of lymphocytes infused in the bone marrow inoculum, referred to as graft-versus-leukemia (GVL). A GVL effect has been reported in animals and man and may be regarded as a form of adoptive immunotherapy (46-48).

Several centers have reported results of bone marrow transplantation in patients with resistant AML transplanted in relapse (49,50). Among these patients, 10% to 20% may survive from 2 to 8 years following transplantation, results that are probably superior to those achieved with alternative therapies (51). Recurrent leukemia was a major cause of treatment failure occurring in 70% of patients within the first 2 years. That all relapses were in recipient cells indicates that even marrow-lethal doses of drugs and radiation could not completely eradicate resistant leukemia. A recent trend is to transplant patients while in their first remission, reasoning that these patients would have a lower leukemia cell burden and that their cells might be more responsive to high doses of drugs and radiation. Several centers have reported 50%-60% disease-free survival with few relapses (52-56). Best results have been achieved in patients < 16 years; older patients do less well. Problems associated with bone marrow transplantation in remission included acute graft-versus-host disease (GVHD) in 25% of patients, interstitial pneumonia in 25%, and chronic GVHD in one-third. Although there is currently considerable enthusiasm for early marrow transplantation in patients with AML, it should be remembered that these are preliminary results in a selected group of young patients. An important and unresolved question is whether early marrow transplantation is superior to conventional therapy. This question is being addressed in several controlled randomized trials currently in progress, and until these data are available, bone marrow transplantation cannot be regarded as routine treatment of AML. Even if these encouraging data regarding marrow transplantation in remission are confirmed, most patients with AML will not be suitable candidates since most are older (median age 50 years) and lack HLA-identical siblings. Alternative approaches include the use of HLA-identical unrelated donors or of partially matched related donors, and alternate sources of hematopoietic stem cells such as fetal liver cells, or marrow autotransplantation using the patients' cryopreserved remission marrow from which leukemia cells have been removed by physical or immune techniques.

One interesting observation reported from both UCLA and Seattle is an anti-leukemic effect of GVHD (46-48). Data from UCLA are shown in figure 5. These data suggest that patients with high-grade GVHD have lower rates of leukemic relapse than those with low-grade GVHD. Since we lack effective therapy for severe GVHD, this potential anti-leukemic effect has not translated into a survival advantage. It does support the concept of a GVL effect of marrow transplantation.

Are there long-term survivors in AML? Some series suggest that there are very few long-term survivors and that all patients eventually relapse. Other studies indicate that 20%-30% of patients remain in remission for 3 to 5 years. Representative data are summarized in table 1 and indicate a 3-year, relapse-free survival rate of approximately 15% (57-61). Some studies show continuing risk of relapse beyond 3 years while others report few late relapses. It is not known which of these contradictory data are correct. A reasonable estimate is that 10%-15% of patients with AML who achieve an initial remission may be cured. In analyzing this long-

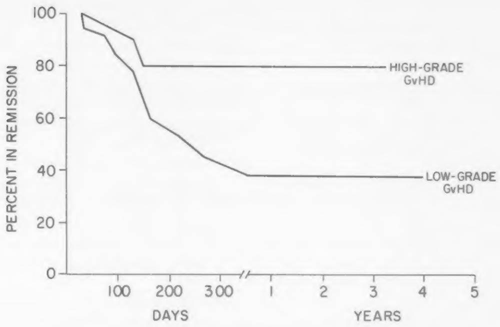

FIGURE 5.—Anti-leukemic effect of high- and low-grade GVHD.

TABLE 1.—Representative data of three-year survival in AML

Center	3-Year survival	
CALGB	16%	(128)
Memorial	18%	(9)
UCLA	24%	(6)
MDA	15%	(5)
Minnesota	21%	(5)
MRC	13%	(3)
SWOG	15%	(22)

term survival data it is important to remember that most patients did not receive modern intensive induction chemotherapy and that better results may emerge when currently treated patients are followed for a sufficient period.

In summary, there has been substantial progress in the treatment of AML during this decade. Seventy percent or more of patients will achieve a remission with intensive two- or three-drug induction chemotherapy. Median remission duration is 9 to 18 months in most studies. There are no convincing data that consolidation, maintenance, intensification chemotherapy, or CNS therapy have had a substantial impact in prolonging remissions. Results with immunotherapy have also been disappointing. Recently, encouraging preliminary results have been reported in young patients with AML undergoing bone marrow transplantation in remission. Finally, a small but definite number of patients with AML, who receive modern therapy, are alive and in remission for more than 3 years and may be cured.

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Treatment of Acute Lymphoblastic Leukemia in Relapse: Efficacy of a Four-Drug Reinduction Regimen[1]

David G. Poplack,[2,*] Gregory H. Reaman,[3] and Robert Wesley[2]

SUMMARY

The experience of 47 similarly-treated patients with acute lymphoblastic leukemia who developed hematologic relapse was reviewed to determine the effectiveness of reinduction therapy. The majority of patients were treated with a four-drug regimen utilizing L-asparaginase, vincristine, daunomycin, and prednisone. This regimen produced second, third, and fourth remissions in approximately 90% of patients, all of whom had experienced relapse while on chemotherapy. Eighteen percent of all patients who achieved second remissions are alive in prolonged second remissions, including four individuals who experienced relapse while receiving maintenance chemotherapy. These encouraging results underscore the efficacy of the four-drug regimen and emphasize the necessity for intensive treatment of recurrent acute lymphoblastic leukemia.

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INTRODUCTION

Despite the considerable improvement which has occurred in the management of children with newly-diagnosed acute lymphoblastic leukemia (ALL), the outlook for patients who have experienced bone marrow relapse or have failed primary induction therapy has been disappointing. Although in many cases it is possible to achieve a second remission, these are usually of short duration, and the likelihood of attaining remission upon subsequent relapse diminishes with each successive relapse (1–4). In the present study, we review our experience in the reinduction of remission in patients with ALL at first relapse and upon subsequent hematologic relapses.

The use of a four-drug reinduction regimen employing L-asparaginase, vincristine, daunomycin, and prednisone (Asp-VDP), resulted in the attainment of second remissions in the overwhelming majority of relapsed patients, some of whom continue in prolonged complete remission, despite having initially relapsed while receiving chemotherapy. In addition, the therapeutic efficacy of this four-drug reinduction regimen did not decline when employed for the treatment of subsequent consecutive relapses.

PATIENTS AND METHODS

Ninety patients, ranging in age from 1 to 20 years, who were treated with similar induction and maintenance therapy during the years 1971–1977 at the Pediatric Oncology Branch of the National Cancer Institute, constitute the current study group. Initial treatment for these patients included induction with POMP (prednisone, vincristine, methotrexate, and 6-mercaptopurine) followed by central nervous system prophylaxis (2400 rads cranial irradiation and intrathecal chemotherapy) and maintenance therapy with 6-mercaptopurine and methotrexate and intercurrent vincristine and prednisone pulses. Some of these patients also received consolidation therapy with L-asparaginase and cyclophosphamide and late intensification (6 months following remission induction) with cytosine arabinoside and daunomycin. The duration of maintenance therapy was 30 months for all patients. Ninety-eight percent of patients treated achieved an initial complete remission.

This report describes the outcome of those 47 patients who experienced hematological relapse. For the

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majority of patients, reinduction therapy included L-asparaginase (*Escherichia coli*), 15,000 IU/M^2 iv on Days 1–5, 8–12, 15–19, and 22–26; vincristine 2 mg/M^2 (maximum 2 mg) and daunomycin 30–60 mg/M^2, iv on Days 8, 15, and 22; and prednisone 40 mg/M^2 po on Days 8–12, 15–19 and 22–28 (fig 1). On this regimen, patients underwent bone marrow aspirations, performed on Days 15 and 22, which were used to determine subsequent daunomycin dosages. On those days, patients whose bone marrow exhibited greater than 5% lymphoblasts received daunomycin at 60 mg/M^2, patients who had 5% or fewer marrow lymphoblasts received 30 mg/M^2 of daunomycin, and patients whose bone marrows were profoundly hypoplastic and exhibited less than 5% blasts had the drug deferred. On the first day of therapy, patients who had previously received L-asparaginase were given a test dose of 100 IU as a 60-min iv infusion during which time they were observed for any hypersensitivity reactions.

The remainder of patients underwent reinduction attempts with treatment regimens different from the one described above. These individuals received various other drug combinations which included either prednisone, vincristine, methotrexate, 6-mercaptopurine, or cytosine arabinoside, cyclophosphamide, 6-thioguanine and methyl CCNU or BCNU. Patients who relapsed at a time following the discontinuation of chemotherapy received POMP reinduction therapy. Otherwise, no specific selection factors could be identified in the determination of the type of reinduction chemotherapy administered. Comparison of the group of patients who received therapy other than Asp-VDP for their attempted reinduction with those patients who received Asp-VDP demonstrated no differences in the median duration of first remission, the type of prior therapy, or initial prognostic factors (initial leukocyte count, age at diagnosis, presence of a mediastinal mass). In all cases, complete remission was defined as a normocellular bone marrow with < 5% lymphoblasts, a normal hemogram and no evidence of extramedullary disease.

In addition to hematological relapse, four patients exhibited central nervous system relapse documented by cytological examination of the cerebrospinal fluid. In combination with systemic chemotherapy these four patients also received intrathecal or intraventricular (via Ommaya reservoir) methotrexate for the treatment of meningeal disease.

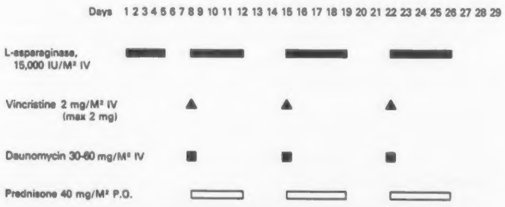
Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
L-asparaginase, 15,000 IU/M² IV
Vincristine 2 mg/M² IV (max 2 mg)
Daunomycin 30-60 mg/M² IV
Prednisone 40 mg/M² P.O.

FIGURE 1.—Asp-VDP treatment regimen.

RESULTS

Outcome of Sequential Reinduction Attempts

The results of successive reinduction attempts for the entire patient population studied are shown in table 1. As noted, second remissions were achieved in 83% of individuals who experienced relapse. Successive reinduction attempts for subsequent consecutive relapses demonstrated a progressive decline in the percentage of patients achieving complete remission. Although third remissions were achieved in 75% of such patients, only 57% achieved a fourth remission.

In contrast, the percentages of remissions achieved for the patients who were treated with successive reinduction courses of Asp-VDP were substantially greater when compared with the entire group (table 2). Ninety-one percent of patients achieved a second remission, 90% a third remission, and 91% achieved complete remission with a third Asp-VDP reinduction attempt. In comparison, analysis of those patients who received other than Asp-VDP for reinduction revealed that only 64% of this group achieved second remissions, 50% attained third remissions and 20% had a fourth remission.

TABLE 1.—Consecutive relapses with corresponding reinduction rates: overall study group

	No. patients	% achieving remission
Total study group	90	98
Achieved first remission	88	
First relapse	47	83
Second remission	39	
Second relapse	32	75
Third remission	24	
Third relapse	21	57
Fourth remission	12	

TABLE 2.—Reinduction rates in patients with multiple relapses: comparison of entire study group with patients receiving Asp-VDP reinduction regimen

	Entire group		Asp-VDP	
Second remission	83%	(39/47)	91%	(30/33)
Third remission	75%	(24/32)	90%	(18/20)
Fourth remission	57%	(12/21)	91%	(10/11)

The relatively small number of patients who received successive reinduction therapy with combination chemotherapy other than Asp-VDP (table 2) and the heterogeneity of reinduction regimens utilized in this group preclude definitive comparison between Asp-VDP and any specific treatment regimen. Nonetheless, it is notable that in those patients treated with Asp-VDP, the incidence of complete remission induction at the time of first relapse approaches that presently being achieved for induction of initial remission (5-11). Furthermore, the reinduction efficacy of the Asp-VDP regimen did not decline when utilized for the treatment of subsequent consecutive relapses in this group of patients. As mentioned previously, comparison of the group of patients who received therapy other than Asp-VDP for their attempted reinduction with those patients who received Asp-VDP demonstrated no differences in the median duration of first remission, the type of prior therapy, or initial prognostic factors. However, it is of particular interest that all the individuals who received Asp-VDP reinduction therapy had experienced their initial hematologic relapse while receiving maintenance chemotherapy. In contrast, five of the 15 patients who received therapy other than Asp-VDP for treatment of their first relapse had relapsed following the discontinuation of initial maintenance therapy. Three of the four patients who presented with extramedullary leukemia in addition to hematologic relapse received Asp-VDP systemic reinduction along with specific CNS therapy and achieved complete remission.

Remission Duration

The patients who achieved complete remissions following relapse were treated with a variety of maintenance regimens. It is therefore difficult to make a meaningful comparison of remission duration between patient groups. However, the remisson duration data for individuals who achieved a second complete remission (fig 2) show that the median duration of second remission was 5 months. Nineteen percent of patients achieved prolonged second remissions. Although one patient died in remission (from infection) at 2 months, six are alive and continue in complete remission with a median followup of 3½ years. Five of these patients have subsequently had their maintenance chemotherapy discontinued. It is of interest that four of these patients had relapsed while receiving maintenance therapy; all four individuals received Asp-VDP as their reinduction chemotherapy.

DISCUSSION

In the present study we reviewed the relapse experience of a group of acute lymphoblastic leukemia patients who were treated with similar initial therapy prior to relapse. Although 83% of this group of patients received a second complete remission, the remission induction rates for subsequent relapses progressively decreased. In contrast, 91% of the patients who had been treated with an intensive four-drug reinduction regimen (L-asparaginase, vincristine, daunomycin, and prednisone) achieved second remissions. Moreover, this latter regimen proved to be equally efficacious in reinducing individuals who experienced second and even third relapses. Although this was a retrospective study, analysis of the subgroup of patients who were treated with Asp-VDP revealed no differences in initial prognostic factors. In addition, all patients who received this regimen had relapsed while currently receiving maintenance chemotherapy. This is of interest because

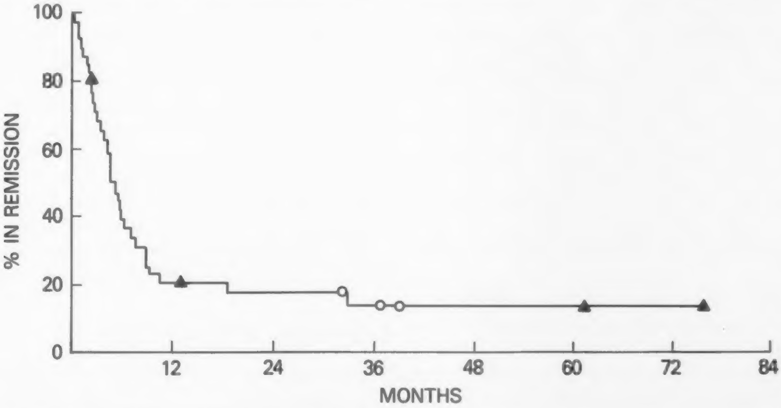

FIGURE 2.—Actuarial remission duration for 39 patients who achieved a second remission. Open triangles (△) identify individuals treated with Asp-VDP. Open circles (O) denote patients treated with other reinduction therapy. Closed triangle (▲) identifies patient who died (from infection) while in second remission.

it has been shown that the rate of reinduction is less in patients who experience relapse while receiving chemotherapy (12). Furthermore, long-term remissions are rarely achieved in this group of patients (13). However, in the current study, three of the 30 patients (10%) in whom a second remission (following relapse on therapy) was achieved using Asp-VDP continue in a prolonged second remission.

In a previous study we demonstrated that the Asp-VDP regimen was highly effective for inducing remissions even in patients who had experienced multiple previous relapses (14). The excellent response rates achievable with this four-drug combination in the treatment of relapsed ALL have been confirmed by other investigators (15). Our experience with this intensive regimen has shown it to be moderately myelosuppressive. However, toxic effects encountered during therapy were not excessive and rarely required alteration in drug dosage. In particular, hypersensitivity reactions to L-asparaginase were rare (14).

The overall response rate for remission induction using Asp-VDP noted in this study, and its apparent efficacy in improving the management of multiple consecutive relapses, is encouraging and emphasizes the need for a prospective study of this regimen in children with recurrent acute lymphoblastic leukemia.

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Optimal Current Treatment of Childhood Acute Lymphoblastic Leukemia[1,2]

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SUMMARY

The treatment of childhood acute lymphoblastic leukemia (ALL) has been remarkably successful over the past 30 years of the chemotherapy era. A substantial proportion of patients, perhaps 40%–50%, are apparently cured with currently available therapy. However, optimal therapy has not yet been devised because of substantial therapeutic failures in the form of relapse and unacceptable side effects. Remission induction therapy is the most settled aspect of therapy. The addition of asparaginase or an anthracycline to prednisone and vincristine is highly effective. Preventive central nervous system therapy is under revision and study, with a variety of approaches likely to prove equally effective. Continuation (maintenance) therapy is in the most need of revision because a majority of therapeutic failures are due to bone marrow relapse—the failure of systemic therapy to prevent emergence of resistant leukemia cells. The optimal duration of therapy has not been established, the relapse rate after cessation of therapy lasting 2–3 years currently being 20%–25%. Bone marrow transplantation during remission shows promise as a therapeutic modality. The most promising development, however, is our increasing understanding of biological subpopulations of leukemia cells. This ultimately may help us develop more effective and specific therapy for childhood ALL.

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The history of the treatment of acute lymphoblastic leukemia (ALL) encompasses several interwoven eras. The era of *discovery* began after the Second World War with the successful use of aminopterin. This was followed over the next 15 years by the introduction of a series of effective agents—corticosteroids, mercaptopurine, cyclophosphamide, and vincristine. Discovery has continued, but at a far less spectacular pace. Of the established front-line agents, only asparaginase and the anthracyclines were discovered after 1960. The era of *combination therapy* began in the late 1950s with the establishment of standards of therapeutic response and of specific experimental designs to test a variety of simultaneous and sequential drug combinations. This era also has seen the introduction of two critical new approaches: the determined aim for cure of ALL instead of palliation and the conceptual development and successful application of therapy for prevention of CNS leukemia. The 1970s were largely an era of *therapeutic refinement*, as investigators spread the concepts and skills of modern therapy, tried permutations of drug schedule and CNS therapy, and attempted to improve results and reduce late effects by using the clinical prognostic category to determine the intensity of therapy. Two promising innovations in this era have been the recognition of leukemia cell features, mainly immunologic, and their relationship to prognosis, and the clinical trials of bone marrow transplantation for treatment of leukemia with curative intent.

The treatment of ALL continues to evolve since optimal therapy has not yet been devised. Current therapy is toxic, prolonged, and, most important, a failure in over one half of all patients. An even higher proportion of adults and other prognostic subsets of patients fail with current therapy. In a real sense, therefore, "standard" therapy for ALL does not exist; all current therapeutic regimens are (or should be) experimental. A description of current therapy should be read with that

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caveat in mind. A commonly-used, though suboptimal, regimen is given in table 1.

CURRENT THERAPY

Remission induction therapy seems well-established with respect to its effectiveness for inducing remission. The addition of asparaginase or an anthracycline to the basic combination of vincristine and prednisone induces remission in all but 5% of patients with ALL. Since there was evidence that, to a point, the intensity of remission induction therapy influenced the duration of remission (1), a current study at St. Jude was designed to test whether administration of four agents during remission induction influenced the duration of remission. So far, patients given the fourth drug have not experienced longer remissions. It would appear, therefore, that a three-drug regimen is optimal with presently available agents.

Preventive CNS therapy is presently receiving the greatest attention of investigators. Although administration of 2400 rads cranial radiation with intrathecal methotrexate remains the standard by which all other forms of CNS therapy must be measured, concern for potential late effects of this therapy (2,3) has led to the development of alternate forms of CNS therapy. The longest successful experience is with intrathecal methotrexate and aggressive systemic therapy (4), which appears to be as effective as irradiation. Other studies of intrathecal therapy, reduced dosage irradiation, and intermediate-dosage methotrexate infusions by vein (5-7) are well underway. It is likely that a variety of forms of CNS prophylaxis will be effective, but it will take time to determine the relative toxicity of each and whether any has specificity for a subgroup of patients.

TABLE 1.—A commonly used, though suboptimal, regimen for treatment of children with acute lymphoblastic leukemia; some alternatives in use and under study are also listed

Remission Induction (4-6 weeks)

- Prednisone 40 mg/m^2/day
- Vincristine 1.5 mg/m^2 weekly
- Asparaginase daily, biweekly or weekly in a variety of dosages

Daunorubicin (and probably Adriamycin) 25 mg/m^2 weekly can be substituted for asparaginase with a similar response

Preventive CNS Therapy

- 2400 rads cranial irradiation with twice weekly intrathecal methotrexate

Intrathecal methotrexate alone, monthly for 3 years and combined with multi-agent systemic therapy (4), as effective

1800 rads may be equally effective for some patients (5,6)

Methotrexate-intravenous infusions of intermediate dosage plus intrathecal dosage — may be effective for some patients (7)

Continuation (Maintenance) Therapy (30-60 months)

- Mercaptopurine 50-75 mg/m^2 daily
- Methotrexate 15-40 mg/m^2 weekly

Periodic pulses with vincristine, prednisone, cytosine arabinoside, Adriamycin or other agents may be of value in some regimens

Continuation therapy during remission has undergone numerous modifications. However, the basic treatment standard continues to be a combination of mercaptopurine and methotrexate. There is no convincing evidence that the addition of other agents during remission significantly prolongs its duration (1,7-9), provided both agents are given, and in maximum-tolerated dosage. This is a phase of therapy which has received the least attention in recent years and yet is responsible for the vast majority of treatment failures in ALL. Most failures are now due to bone marrow relapse which is attributable to the development of resistance to the agents given during remission. Above all, this is the phase of therapy that most needs a fresh approach and the courageous trial of new ideas. At St. Jude we are re-examining the use of elective sequential therapy to prevent emergence of resistant leukemia cells. Instead of single agents, we are testing the value of pairs of agents each given for several months. This study has just started and we have no data to report.

Cessation of therapy has arbitrarily been done after 28 to 36 months of initial complete remission at St. Jude; subsequently, 75%-80% of patients remain in remission indefinitely. As a result of this observation, we have developed an operational definition of cure for this disease, that is, that the patient remain in remission for 4 years after cessation of therapy (10). Longer durations of therapy are being tested by the cooperative groups. Although the 20%-25% relapse rate in our studies is not acceptable, we believe prolonging the same therapy most likely will merely shift the time of relapse of these patients without reducing the number of relapses. Because of the relatively high frequency of testicular relapse in boys after cessation of therapy, we now routinely perform bilateral testicular biopsies at the time therapy is to be discontinued.

Bone marrow transplantation for ALL has generated new interest with the promising results of transplanting patients during remission (11). By transplanting patients during a second or subsequent remission, many of the early deaths due to infectious and hemorrhagic complications may be avoided. If the current trend continues, the proportion of long-term survivors will be as many as 30% to 50% compared to the 7% to

15% among patients transplanted mainly during relapse.

ACTIVITIES FOR THE 1980s

During the next decade we may expect further refinement of the activities noted above: more information on the effectiveness and side effects of the various forms of preventive CNS therapy now being tested; epidemiological studies of late effects of all kinds in the increasing number of long-term survivors; completion of current studies of the optimal duration of therapy by the Childrens Cancer Study Group and the Cancer and Leukemia Group B; and continued studies of ways to use current agents more effectively. The latter remains an important exercise—it is remarkable that we are still seeking the optimal schedule and dosage for the use of methotrexate in ALL more than 30 years after its introduction. There will be continued attempts to improve the results of treatment of patients with poor prognostic features. Thus far, the rationale has been that this more resistant form of the disease requires more intensive therapy with more drugs and higher dosages. However, this approach has not improved the results. More effective therapy may depend upon the emergence of useful clues from studies of the biological basis of resistance (12); this is more likely to occur now because of the relative ease of identification of some subclasses of leukemia cells. An empirical approach is unlikely to be successful without a radical departure from current regimens or the discovery of a new and highly effective drug. With all the focus on patients with features associated with a poor prognosis, insufficient attention has been paid to the fact that we do not understand the biological basis for the failure of patients without poor prognostic features. At least one third of "standard risk" patients will develop a bone marrow relapse despite their designation as "common" ALL. It is critical that we learn whether these relapses are avoidable by the more optimal use of current agents or whether resistance is innate.

There are two new agents with some promise for treatment of ALL. Studies of the combination of the epipodophyllotoxin VM-26 and cytosine arabinoside have shown considerable promise in patients resistant to other forms of treatment (13). In fact, this combination is so promising that it is being incorporated into our new front-line studies for ALL. Another agent that bears watching, both because of its early promise and because of its unique mode of action, is deoxycoformycin (14). This agent is an adenosine deaminase inhibitor that specifically attacks lymphoid cells with little apparent effect on other bone marrow-derived cells. It is too early to determine whether this specificity will hold up in large trials.

The most urgent need of the 1980s, in my view, is a method for detecting minimal residual disease in the bone marrow of patients who are in remission of ALL. If we had the capacity for detecting one leukemia cell in a thousand or a million or more, we would be able to measure the effectiveness of therapy given during remission. Currently, of course, we must wait for relapse to occur and we have no clue as to when resistance may have developed. Detection of minimal residual disease is within reach for ALL because of the rapid development of our ability to raise quantities of monovalent antisera with hybridomas and of technology such as flow cytofluorimetry with cell separation capability.

Advances in the treatment of ALL, particularly in childhood, have been truly remarkable over the past 30 years. The eras of discovery and combination therapy continue but are overshadowed by the era of therapeutic refinement. One hopes that the 1980s will see a new era in the treatment of ALL: the era of the biological specificity.

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L-Asparaginase as a Single Agent in Acute Lymphocytic Leukemia: Survey of Studies From Childrens Cancer Study Group[1]

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SUMMARY

A series of studies were undertaken by the Childrens Cancer Study Group between 1968 and 1972 to evaluate the optimum schedule and dosage of L-asparaginase as a single agent in induction and maintenance of children with previously treated acute lymphocytic leukemia. Six different dosages of enzyme were administered (3600–840,000 IU/4 wk/m^2). The optimal dose of L-asparaginase was found to be 12,000 IU/m^2 given three times per week for a total dose of 144,000 IU/m^2. Sixty-three percent of children treated with this dosage achieved a bone marrow remission. The intramuscular method of administration is as effective and produced less significant sensitivity reactions than the intravenous method. Reinduction, after an unmaintained remission, with L-asparaginase was possible in 32% of the cases. L-Asparaginase used as a maintenance agent did not result in a significant increase in remission duration when compared to no maintenance therapy. However, patients who received L-asparaginase maintenance had a median remission duration of 66 days as compared to 41 days for the no-maintenance group.

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L-Asparaginase, an enzyme produced by *Escherichia coli,* catalyzes the hydrolysis of asparagine and has been shown to be an effective agent in the treatment of acute lymphoblastic leukemia (ALL) in children (1–3). At the present time, L-asparaginase is most commonly used in combination with prednisone and vincristine for initial induction (4) and re-induction of ALL (5).

Childrens Cancer Study Group conducted a series of clinical investigations designed to study L-asparaginase as a single agent to determine the optimum dose and schedule of administration. This report summarizes these studies, some of which have been previously reported (5–8).

MATERIALS AND METHODS

A series of protocols were initiated by the Childrens Cancer Study Group in 1968 to determine the most effective method of administration and dosage of L-asparaginase for previously treated children with ALL in bone marrow relapse (CCG-806, CCG-901, CCG-002). L-Asparaginase was provided in 2000- and 10,000-unit vials by the Bayer Company, whose representative in the United States is Delbay Pharmaceutical, Inc., Bloomfield, New Jersey. L-Asparaginase was used after dilution with 0.9% NaCL solution (2–4 ml) without preservation. The enzyme was immediately injected intravenously or intramuscularly. Patients were eligible for the protocols if they had not received L-asparaginase before and if they had relapsed at least once. The diagnosis of acute leukemia (lymphoblastic, stem cell, undifferentiated) was established if the bone marrow aspirate contained more than 25% blast cells. Those patients treated concomitantly with 6-mercaptopurine (6-MP) must have been considered resistant as evidenced by either relapsing on 6-MP maintenance or failing induction with 6-MP. Criteria for evaluating chemotherapy in acute leukemia in children as adopted by Childrens Cancer Study Group (CCSG) in September 1963, were used in these studies (9). Patients who developed CNS leukemia during induction therapy were treated with either intrathecal methotrexate or radiation therapy. Patients were removed from the study if they failed to enter remission or if they had uncontrollable toxicity.

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Initial observations that were mandatory were physical examination, cbc, bone marrow aspirate, BUN, uric acid, SGOT, and SGPT or OCT. The following initial observations were optional and drawn if symptoms and signs warranted: bilirubin and protein electrophoresis, cholesterol, triglycerides, phospholipids, calcium, phosphorus, and coagulation studies. Subsequent observations during the induction period included a bone marrow on Days 14 and 28. BUN and uric acid were repeated on Days 3 and 5 and liver function tests were repeated at the end of induction.

During the maintenance phase, a physical examination was performed at least once every 2 weeks. Bone marrow and liver function were to be examined once a month. In studies CCG-806 and CCG-901, skin tests with 0.5 IU of L-asparaginase in 0.1 cc saline intradermally with a control using saline in the appropriate arm was used. During the reinduction phase, all observations were scheduled as in the initial induction period. The skin test was to be repeated, and regardless of whether the test results were negative or positive, each child had to be desensitized.

Summarized in table 1 are dose schedules of L-asparaginase for the three studies conducted by CCSG. The overall purposes of these three studies were: 1) to evaluate two different routes of administration (iv vs im), 2) to evaluate optimum dose for induction, 3) to determine the value of maintenance therapy with L-asparaginase, and 4) to determine whether reinduction with L-asparaginase is effective. The first study (CCG-806) was initiated in 1968 using a total induction dose of 168,000 IU/m^2 of L-asparaginase given during a 4-week period. There was a randomization between iv and im methods of administration. The drugs were given intermittently (three times a week). Patients who achieved a remission were then randomized to no-maintenance chemotherapy versus continued L-asparaginase. The subsequent protocol (CCG-901) was conducted from 1969 to 1970 using the same intermittent L-asparaginase schedule. In this study patients were randomized to receive either L-asparaginase or L-asparaginase plus 6-MP. A total dose of 840,000 IU/m^2 iv was used over a 4-week period. All patients achieving a remission were followed unmaintained. Those patients who subsequently relapsed were reinduced with their previous induction regimen. The third study (CCG-002) ran from 1971 through 1972. During the first year of the study, previously treated ALL patients were induced with L-asparaginase and 6-MP and randomized to either 12,000 IU/m^2 three times a week or 300 IU/m^2 three times a week. During the second year of patient accrual, patients were randomized to 6000 IU/m^2 three times a week versus 3000 IU/m^2 three times a week.

In the CCG-002 series all patients were 6-MP resistant, as previously defined. 6-MP was given in combination with the L-asparaginase to reduce sensitivity reactions. This approach was used based on study CCG-901 which had shown that the combination of L-asparaginase with 6-MP, in patients resistant to 6-MP, significantly reduced the incidence of serious sensitivity reactions.

TABLE 1.—CCSG protocols using L-asparaginase for previously treated ALL in children (1968–1972)

Year	Study no.	Chairman	Schedule (IU/m^2)	Route	Total dose induction (4 wks)
1968-69	CCG-806	Nesbit	12,000 M.W. 18,000 F.	iv or im	168,000 IU/m^2
1969-70	CCG-901	Nesbit	60,000 M.W. 90,000 F.*	iv	840,000 IU/m^2
1971-72	CCG-002	Ertel	12,000 M.W.F.*	im	144,000 IU/m^2
1971-72	CCG-002	Ertel	6,000 M.W.F.*	im	72,000 IU/m^2
1971-72	CCG-002	Ertel	3,000 M.W.F.*	im	36,000 IU/m^2
1971-72	CCG-002	Ertel	300 M.W.F.*	im	3,600 IU/m^2

*Received 6-MP to reduce sensitivity reaction.

RESULTS

Comparison of Intramuscular and Intravenous Routes of L-Asparaginase

CCG-806 utilized a total induction dose of 168,000 IU/m^2 in 4 weeks. Patients were randomized between iv versus im administration of the enzyme. Thirty out of 87 (34%) achieved a marrow remission (M-1) with im administration (table 2). Hepatic, renal and hematologic toxicity was comparable for the two groups. Four patients had significant renal toxicity, three on iv and one on im administration. Ten patients had significant hepatic toxicity with hyperbilirubinemia or liver failure. Four of these ten patients received the iv schedule of whom two experienced significant liver failure and hepatic coma. In the group randomized to receive the im route, six patients had significant hyperbilirubinemia but no patients experienced hepatic coma. The most significant toxic effect of L-asparaginase was the sensitivity reactions. Eighteen patients had an anaphylactic reaction, all of whom received the iv schedule of administration. There were five such reactions during the second week of treatment, 11 during the third week, and two during the fourth week.

Complications were also compared between the two methods of administration. For non-fatal disease-related complications there was no significant difference between the incidence of infections (21 iv and 22 im) or bleeding episodes (11 iv and 10 im) for the two groups. This lack of difference between the two groups is also true for disease-related deaths. There were 21 deaths in the intravenously treated group, four due to bleeding, 13 due to infections and four unknown; and 12 deaths with the intramuscular group, three due to bleeding and nine due to infections.

After achieving a remission, 24 patients, 14 in the iv group and ten in the im group, all of whom received a total dose of 168,000 IU/m^2 over 4 weeks, were observed for length of unmaintained remission. The median duration of unmaintained remission was 37 days for the iv group and 44 days for the im group (table 3).

Optimum Induction Dose

Between 1968 and 1972, six different dosages of L-asparaginase were evaluated as to their ability to induce remission in patients with previously treated ALL (table 2). The 10% induction rate with the low dose (3600 IU/m^2) increased to 63% when the total dose was

TABLE 2.—Remission induction with L-asparaginase for previously treated ALL in children CCSG protocols (1968–1972)

Patients/year	Total dose L-ASP induction/4 wk	Route	M-l/patients entered	% remission
CCG-901	840,000 IU/m^2	iv	26/81	32
1969-70	840,000 IU/m^2*	iv	26/78	33
CCG-806	168,000 IU/m^2	iv	30/87	34
1968-69	168,000 IU/m^2	im	26/77	34
CCG-002 1971-72	144,000 IU/m^2*	im	20/32	63
CCG-002 1971-72	72,000 IU/m^2*	im	69/129	54
CCG-002 1971-72	36,000 IU/m^2*	im	13/37	35
CCG-002 1971-72	3,600 IU/m^2	im	2/21	10

*Received 6-MP to reduce sensitivity reaction.

TABLE 3.—Duration of unmaintained remission after im or iv L-ASP

Dose-induction	Route	No. of patients	Remission duration days		
			Mean	Median	Range
168,000 IU/m^2/4 wk	iv	14	44	37	35-91
	im	10	44	44	24-137

increased to 144,000 IU/m². At higher dosages of L-asparaginase (168,000 IU/m² and 844,000 IU/m²), there is no further increase and even a decrease to 34% and 32%, respectively (fig 1).

After successful induction, 73 patients were followed unmaintained. There is no significant difference in the duration of unmaintained remission when comparing the various doses given during induction (table 4).

Reinduction

As part of this series of protocols using L-asparaginase, patients who had been followed in unmaintained remission and subsequently relapsed were then eligible for reinduction using L-asparaginase. Sixty-five patients underwent reinduction, 24 of them with 168,000 IU/m² and 41 after receiving 840,000 IU/m². Seven out of the 24 with the lower dose and 14 out of the 41 at the higher dose achieved a remission (table 5).

Maintenance

In protocol CCG-806, which used a total dosage of 168,000 IU/m², an additional objective of the study was to determine the value of maintenance therapy with L-asparaginase. Fifty patients who had achieved remission in induction were randomized to continued treatment with L-asparaginase or no further therapy. The median remission duration was 66 days for the maintenance group and 41 days for the no-maintenance group.

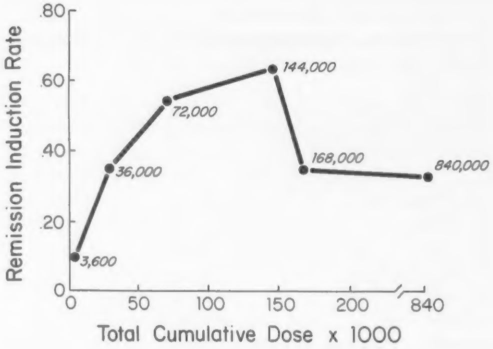

FIGURE 1.—Induction rates using L-asparaginase in previously treated childhood acute lymphoblastic leukemia.

DISCUSSION

With current chemotherapeutic combinations, including the use of L-asparaginase, it is possible to achieve an initial remission in approximately 95% of all pediatric patients diagnosed with ALL (4). The lack of significant marrow toxicity makes it an excellent drug to be used in combination therapy. But the enzyme is associated with a wide range of other toxic effects which include significant anaphylactic reactions, pancreatitis, coagulopathy, hypocomplementemia, and

TABLE 4.—Duration of unmaintained remission after L-ASP induction

Dose 4 wk/m²	No. of patients	Duration of unmaintained remission (days) Mean	Median	Range
840,000 IU	26	60	58	14–124
168,000 IU	24	48	41	24–139
144,000 IU*	4	70	57	49–128
36,000 IU*	11	51	54	21–56
3,600 IU*	8	49	49	21–128

*Received 6-MP to reduce sensitivity reactions.

TABLE 5.—Reinduction using L-ASP after unmaintained remission

Total dose of L-ASP/4 wk	No. of patients	Remission	%
168,000 IU/m²	24	7	29%
840,000 IU/m²	41	14	34%

thrombotic episodes (10–14). In general, the toxic effects are dose-related and it is thus important to determine a safe and effective dose schedule of L-asparaginase administration.

CCSG successfully conducted a series of clinical trials beginning in the late 1960s designed to investigate the effectiveness of L-asparaginase for the treatment of childhood ALL. L-Asparaginase used as a single agent was found to be effective in producing second or subsequent remissions in 30%–65% of the patients treated. Moreover, L-asparaginase was found to be an active agent for reinduction of patients previously induced and/or maintained with the enzyme.

To date, there is no generally accepted dose, schedule or route of administration for L-asparaginase. The studies reported here suggest that intramuscular administration is less toxic yet as effective as the iv route. Of the dosages investigated by CCSG which ranged from 300 IU/m^2 to 60,000 IU/m^2 the most effective dose with regard to remission induction was 12,000 IU/m^2 three times a week, for a total dose of 144,000 IU/m^2 in 4 weeks. Doses above this level were found to be less effective, resulting in a lower remission rate. The reason for this reduction in the remission rate is not immediately apparent. Although the serious toxic effects were more common at the higher doses, the differences in the induction rates do not appear to be the result of an excess in drug-related mortality or morbidity.

Patients who received L-asparaginase as a maintenance agent were found to have longer disease-free intervals than comparable patients who received no maintenance therapy. Although this was not a statistically significant difference, the median remission duration for the L-asparaginase group was 66 days as compared to 41 days for the non-maintained group. These data suggest that incorporation of L-asparaginase with other maintenance chemotherapeutic regimens is warranted. Moreover, the data also suggest that patients who receive L-asparaginase during maintenance remain responsive to the enzyme when subsequently utilized for reinduction. The rationale for therapeutic regimens such as those introduced by Capizzi (15) or maintenance reinduction programs using L-asparaginase appear to be substantiated by the early work of Childrens Cancer Study Group.

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Appendix 1.—Principal investigators Childrens Cancer Study Group

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Children's Hospital National Medical Center George Washington University Washington, DC	Sanford Leikin, M.D.	CA-03888
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Children's Hospital of Pittsburgh University of Pittsburgh Pittsburgh, PA	Vincent Albo, M.D.	CA-07439
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Children's Orthopedic Hospital and Medical Center University of Washington Seattle, WA	Ronald Chard, M.D.	CA-10382
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University of Minnesota Health Sciences Center Minneapolis, MN	Mark Nesbit, M.D.	CA-07306
Children's Memorial Hospital Northwestern University Chicago, IL	George Honig, M.D.	CA-07431
University of Utah Medical Center Salt Lake City, UT	Eugene Lahey, M.D.	CA-10198
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Children's Hospital University of Louisville Louisville, KY	Donald R. Kmetz, M.D.	—
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APPENDIX 1.—Principal investigators Childrens Cancer Study Group—Continued

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Clinical Pharmacology of Daunorubicin

Robert S. Benjamin[1,*]

Studies of the clinical pharmacology of daunorubicin have been greatly aided by the specific fluorescent characteristics of the anthracycline antibiotics. The conjugated ring structure in the aglycones imparts to these drugs a characteristic orange-yellow fluorescence which is maximized by excitation at 470 nm with fluorescence at 585 nm. Essentially, all of the major clinical pharmacology studies on daunorubicin originate from the laboratory of Dr. Nicholas Bachur at the National Cancer Institute, Baltimore Cancer Research Center. Initial studies defined the advantage of fluorescent methodology over the use of randomly tritiated daunorubicin (1). Clinical pharmacology studies of daunorubicin as measured by total plasma fluorescence were then carried out by Alberts et al (2) in patients with solid tumors. These studies revealed that after initial rapid distribution, the drug had a prolonged plasma half-life. The studies were extended by Huffman et al (3), who defined daunorubicin metabolism for the first time. Daunorubicinol had previously been described as the major metabolite of daunorubicin by Bachur (4). In the Huffman study the pharmacokinetics of daunorubicinol and chromatographically isolated daunorubicin were defined (3). Daunorubicin was cleared in a biphasic pattern with a very short initial half-life followed by a mean half-life of 18.5 hours. Daunorubicinol rapidly exceeded daunorubicin in total concentration, representing 40% of total plasma fluorescence at 30 minutes and 60% at 4 hours.

The technique used to identify daunorubicin and daunorubicinol in that study involved acid hydrolysis of the plasma extract with conversion of daunorubicin and daunorubicinol to their respective aglycones. Since significant amounts of aglycones had not been detected following dichloromethane extraction in the studies by Alberts et al (2), it was assumed that all of the aglycones detected after hydrolysis represented conversion from the parent glycosides (3). A new metabolite, coded D5, was described in the plasma of patients treated in Huffman's study (3). This metabolite had an R_f intermediate between those of daunorubicin aglycone and daunorubicinol aglycone following the hydrolysis extraction procedure. The material increased slowly in plasma over the first 8 hr to reach levels higher than those of unchanged daunorubicin after 12 hr. With the development of a non-destructive plasma extraction technique for Adriamycin (5), it was possible to determine that the metabolite D5 was an aglycone produced in vivo[2].

The major step in daunorubicin metabolism involves the reduction of the ketone at carbon 13 to a hydroxyl group forming daunorubicinol (2–4,6–7). The class of aldo-keto reductases involved in this metabolic reaction have been extensively studied by Bachur (6). The second major step in daunorubicin metabolism is the reductive cleavage of the glycosidic bond yielding 7-deoxy aglycones. The major metabolic pathways of human metabolism of daunorubicin have been elegantly worked out from studies of metabolites in human urine by Takanashi and Bachur (7). Following reduction of daunorubicin to daunorubicinol, the next major step is the reductive cleavage to form 7-deoxydaunorubicinol aglycone with subsequent 4-0-demethylation and conjugation to a 4-0-sulfate or glucuronide. Much smaller amounts of daunorubicinol are hydrolyzed by simple hydrolysis to daunorubicinol aglycone. Small amounts of daunorubicin could undergo reductive cleavage to 7-deoxydaunorubicin aglycone prior to reduction to 7-deoxydaunorubicinol aglycone. Small amounts of the latter compound also could undergo glucuronide conjugation at carbon 13 without 4-0-demethylation (7).

Tissue distribution of daunorubicin was studied in a single patient. Levels were highest in the kidney, pancreas, and liver; lowest in fat; and slightly higher in skeletal muscle, lung, heart, and spleen. Daunorubicin was never the principal tissue component, although it was equivalent in concentration to metabolite D5 in pancreas. Daunorubicinol was the principal metabolite in liver. Metabolite D5 was the principal component in most tissues, ranging in relative concentration from 35% in liver and pancreas to 77% in heart. Only in cardiac muscle was this metabolite so much more prominent than the others (3).

Urinary excretion of daunorubicin accounted for 23% of the administered dose. The primary metabolic

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species in the urine was daunorubicinol, particularly after the first 24 hours. Daunorubicinol accounted for almost two thirds of the total amount excreted, whereas daunorubicin accounted for about one third. Metabolite D5, and another similar metabolite, D7, were detected in much lower concentration (3). It should be noted that the urinary excretion of daunorubicin is considerably higher than that of Adriamycin. Using an identical procedure, we detected less than 6% of an administered Adriamycin dose in the urine (8). In contrast to the findings with daunorubicin, Adriamycin and not Adriamycinol was the principal component of the material excreted.

Daunorubicinol is the principal plasma species following daunorubicin administration. Whether conversion of daunorubicin to daunorubicinol represents activation cannot be determined; however, it is clear that daunorubicinol is an active inhibitor of DNA and RNA synthesis (9). It is less effective at cellular penetration than is daunorubicin, perhaps accounting for its lower activity in experimental systems (9,10). It is, in fact, a chemotherapeutic agent which has been used in France under the name of Duborimycine (11).

Daunorubicin toxicity is similar to that of other anthracyclines. Dose-limiting toxicity during induction chemotherapy for acute leukemia is stomatitis which occurs in about 40% of patients. Acute febrile drug reactions occur in about 50% of patients and probably represent reactions to histamine release. Alopecia is universal. Extravasation causes sloughing. Nausea and vomiting occur in about 50% of patients (12). When doses are reduced to give tolerable myelosuppression during maintenance chemotherapy, stomatitis essentially disappears as a toxic effect on a 3-day schedule. This is similar to what is seen with rubidazone (13), but quite different from what is seen with Adriamycin on a 3-day schedule (14). The relatively increased myelosuppressive toxicity, as compared with mucosal toxicity for daunorubicin and rubidazone, and as compared with Adriamycin, may allow much higher relative doses of these drugs to be used during single agent induction therapy of acute leukemia. This may account for the relatively increased activity of these drugs as single agents in the treatment of acute leukemia.

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L-Asparaginase: Discovery and Development as a Tumor-Inhibitory Agent

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SUMMARY

The early observations identifying the tumor-inhibitory agent in guinea pig serum as L-asparaginase are summarized, together with further studies on the relationship of L-asparagine synthetic capacity of lymphoma and other cells to sensitivity to the L-asparaginase. The combination of characteristics which render L-asparaginase a uniquely effective tumor-inhibitory enzyme are discussed.

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The story of the discovery of the tumor-inhibitory properties of L-asparaginase begins with an observation made by John G. Kidd (1) in 1953. In attempting to increase the effectiveness of hetero-antisera as inhibitors for standard transplantable tumors in mice, Kidd administered fresh guinea pig serum, and observed that certain lymphoid tumors (particularly 6C3HED) which normally grew to kill their hosts underwent rapid and often complete regression. Most surprisingly, however, regression also occurred in controls to which guinea pig serum was given alone. Kidd went on to test various animal sera and found that of these guinea pig serum was unique in having tumor-inhibitory activity (2).

Because the high activity of hemolytic complement in guinea pig serum was well known, it seemed probable that this was responsible for Kidd's observations. Indeed, many years later it was shown by Kassel and co-workers (3) that complement could indeed have an antitumor effect, causing the rejection of autochthonous thymic lymphomas in AKR mice.

But in the immediate context of the cell systems used by Kidd, certain experiments suggested that another component of guinea pig serum might be responsible for tumor inhibition. Other animal sera which had high complement activity were inactive. Furthermore, as guinea pig serum-resistant variants of 6C3HED were obtained (following subcurative doses of guinea pig serum), they did not differ from the original line in their ability to induce cytotoxic antibody in homologous hosts or in sensitivity in vitro to homologous antiserum combined with guinea pig serum (4).

A search was therefore undertaken to identify distinctive components of guinea pig serum which differed from sera which did not have antitumor activity. One such component was the enzyme L-asparaginase which had been studied by Clementi (5) in the 1920s from the point of view of comparative biochemistry. In the species he tested it was found that L-asparaginase was present in the blood only of the guinea pig although it was present in the liver and various organs of many others. We now know that blood L-asparaginase is characteristic of the *Cavioidea*, the superfamily to which the guinea pig belongs (6).

L-Asparaginase is an easy enzyme to assay chemically, and fortunately, a sensitive bioassay for tumor-inhibitory activity was available using 6C3HED. Cells implanted subcutaneously in the flanks of C3H mice caused palpable tumors 7-12 days later, depending on the dose inoculated. Inhibitory activity was measured by the delay in tumor appearance compared with untreated controls. A series of experiments was therefore undertaken to examine enzyme properties in relation to tumor-inhibitory activity. In salt solubility pattern, stability to heat, and pH change, a good correlation was obtained between the two (7).

The bioassay also permitted the design of purification procedures in which at various steps enzyme activity could be compared with that of tumor inhibition. Salt precipitation, electrophoresis on starch blocks, and DEAE cellulose chromatography were performed successively and on each occasion a good correlation between the two activities was obtained. A final increase in specific activity of 88 times was achieved (8).

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A further line of evidence was being explored at the same time as the experiments just described. It was found that 6C3HED cells could be cultured in a medium as simple as the basal medium of Eagle with 10% horse serum. Cultures took a period of several weeks in which the cell count dropped to about 1% of that inoculated before they became established, but when this was done they proliferated vigorously and could then be maintained indefinitely. When such proliferating lymphoma cells were inoculated in vivo they proved to be totally insensitive to guinea pig serum, and in prolonged animal passage maintained this characteristic. Examination of the formula for the medium showed that it lacked L-asparagine. When this amino acid was added, the lymphoma cells proliferated vigorously from the initiation of culture and on reinoculation into animals maintained sensitivity to guinea pig serum. We thus demonstrated the correlation between L-asparaginase dependence and guinea pig serum sensitivity and, contrariwise, L-asparagine independence and guinea pig serum resistance (6,9).

The final proof that L-asparaginase was the tumor-inhibitory agent of guinea pig serum was furnished by Wriston and Yellin (10) who isolated the enzyme to homogeneity as judged by immunoelectrophoresis and showed that it was strongly inhibitory to Lymphoma 6C3HED.

BACTERIAL ASPARAGINASES AND HUMAN THERAPY

Mashburn and Wriston (and other workers) (11–14) also examined asparaginases from sources other than guinea pig serum; they found that *Escherichia coli* yielded preparations which inhibited tumors, but other bacterial asparaginases were either less active or completely inactive. In fact *E. coli* was later shown to possess two enzymes, one constitutive (EC1) and another induced by anaerobiosis (EC2); only the latter was tumor-inhibitory (15,16). Tumor-inhibitory enzymes have since been isolated from a number of other bacteria (17), notably because of its therapeutic use from *Erwinia carotovora* (18).

Distinct characteristics were shown to be required for an asparaginase to be tumor-inhibitory (19). A high affinity for L-asparagine was required (the *E. coli* enzyme with tumor-inhibitory activity had a Km of 1.25×10^{-5}M, the inactive enzyme about 5×10^{-3}M). Secondly, it was necessary for the enzyme to persist sufficiently long in the circulation of the recipient animal. Turnover of some bacterial asparaginases is greatly modified by infection of mice with the lactic dehydrogenase-elevating virus, which is carried in many transplanted tumors (19,20). Indeed, if it were not for this fortunate coincidence the effectiveness of *E. coli* asparaginase might not have been detected. The enzyme is without effect in animals with recently inoculated lymphoma cells; its clearance half-life in normal mice or those within 2 days of tumor cell implantation is 2.6 hours; while in tumor-bearing animals with established virus infection it reaches 24.1 hours (16). However, the clearance rate of L-asparaginase varies between species and in humans the half-life of EC2 is from 11.2 to 22.6 hours, depending on the preparation used, rendering it suitable for the therapeutic trials (21).

Before such trials were undertaken substantial evidence had accumulated from animal experiments on the spectrum of tumor sensitivity. In mice 28 of 109 lymphomas induced in various ways proved to be sensitive, as did many feline lymphomas, lymphomas of dogs and also mastocytomas (22,23). Very few nonlymphoid tumors proved to be sensitive. Development of L asparaginase-resistant variants also become apparent in the various mouse lines examined after treatment with subcurative doses, as had been the case in 6C3HED.

Mice proved to be highly resistant to toxicity from L-asparaginase and doses as high as 2,000,000 U/kg could be administered (24). However, toxic effects of numerous kinds became obvious in other species, indicating a lack of absolute specificity against the tumor cells (25).

The first clinical trial of L-asparaginase used guinea pig serum in a terminal leukemia patient and provided some indication that it was effective (26).

Highly purified *E. coli* L-asparaginase (EC2) became available in quantity soon afterwards as a result of considerable effort by individual laboratories and by the pharmaceutical industry, and striking remissions were reported in many of the patients with acute lymphocytic leukemia who received the enzyme (27,28). However, these remissions proved to be relatively short-lived, with a median of 122 days (29). Very few responses were found with other neoplastic conditions. Toxicity was also observed and a wide spectrum of reactions including coagulation defects, pancreatitis, fatty liver, neurological changes and hypersensitivity have been recorded (30,31).

Clearly, L-asparaginase was not able to completely eliminate the tumor cells but with continued trials it proved to have definite clinical advantages and effectiveness when used as a component of multiple-drug treatment schedules for acute lymphocytic leukemia. This is the subject of other presentations in this symposium.

Mechanism of Action

Early experiments described above showed that guinea pig serum-sensitive tumors were L-asparagine dependent (7,9). In its absence, in vitro protein synthesis was sharply reduced within 15 minutes (32). L-Asparagine dependence was shown to be due to a very low synthetic capacity for the amino acid in dependent cells as compared with independent cells (33). Furthermore, in protein synthesis endogenous L-asparagine was used preferentially over the exogenous amino acid (34).

An enzyme synthesizing L-asparagine from aspartic acid was demonstrated by Paterson and Orr (35) in an L-asparagine independent variant of the Jensen sarcoma, using pH5 precipitates from cell homogenates; it required L-glutamine as the amide donor, ATP, and Mg^{++}. The synthetase was undetectable in the L-asparagine dependent line of Jensen sarcoma. Assays of lymphoma cells soon confirmed this observation and showed a correlation (at least in mouse cells) between low synthetase activity and L-asparaginase sensitivity (34). A similar correlation has not been demonstrated in human leukemia cells. Most leukemic cells show no synthetase activity irrespective of their eventual response to treatment (36). Perhaps sensitive and insensitive lymphoma cells differ in the ease with which synthetase can be induced.

The question of the final lethal route of susceptible cells deprived of L-asparagine is also still undecided. The possibility that an overall inhibition of protein synthesis is responsible appears unlikely. Sensitive tumor cells exhibit severe cytotoxicity within 2 hours when treated with L-asparaginase in vivo as Kidd (1) showed in his earliest observations, but they survive for considerably longer periods when protein synthesis is almost completely inhibited by incubation in buffered salt-glucose solutions in the absence of amino acids. Furthermore, other cells, such as liver cells after hepatectomy, are prevented from dividing by L-asparaginase, but show no apparent cytotoxicity and eventually resume growth (37). One possible explanation for its destructive effect on lymphoma cells is the intracellular accumulation of aspartic acid which occurred to a striking degree after L-asparagine deprivation in dependent cells and reached levels 12 times above normal (34). It has been postulated that since L-aspartic acid is a requirement for L-asparagine synthesis, release of biosynthetic feedback might be expected to increase its concentration as that of L-asparagine falls (34). Lack of other control of aspartate synthesis would lead to the massive accumulation of aspartate which is observed. L-Aspartate at high concentration may be inhibitory to basic energy producing mechanisms of the cell and thus cause rapid cytotoxicity.

CONCLUSION

The activity of L-asparaginases in inhibiting lymphomas appears to be due to several unusual coincidences. The L-asparaginases are hydrolytic enzymes that require no co-factor and have high affinity for their substrate. Certain lymphoma cells appear to have an unusually (but not necessarily unique) low level of L-asparagine synthesis in the face of the powerfully depleting action of the enzyme. Finally, a rapidly lethal sequence of uncontrolled changes occurs in leukemic cells following isolated L-asparagine deprivation. L-Aspartate accumulation may be important in this. Whether other enzymes and substrates have properties with a comparable advantage for therapy remains to be determined. A number of these are currently under study and it is to be hoped that L-asparaginase will not continue to be unique.

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Asparaginase-Methotrexate in Combination Chemotherapy: Schedule-Dependent Differential Effects on Normal Versus Neoplastic Cells[1]

Robert L. Capizzi[2,*]

SUMMARY

In a variety of cell culture and in vivo experiments with normal and tumor-bearing animals, the antecedent or simultaneous use of protein synthesis inhibitors with antimetabolites or alkylating agents will significantly attenuate the cytotoxic effects of the latter. The protein synthesis inhibitor asparaginase shares this potential. In murine leukemia L5178Y which is sensitive to both asparaginase and methotrexate (MTX), the prior use of asparaginase or the simultaneous administration of both drugs results in subadditive effects. In tumor-bearing mice, multiple courses of sequential MTX followed by asparaginase cured 55% of the leukemic mice whereas the converse sequence cured none. Partial explanation for this pharmacologic antagonism includes asparaginase-induced decrease in cellular uptake of MTX and delay in cell cycle traverse. It is of importance to recognize such pharmacologic antagonism for the proper design of clinical trials. Studies with human leukemic lymphoblasts suggest that the optimal time interval between asparaginase and a subsequent dose of MTX was 9–10 days. A 24-hour interval between methotrexate and a subsequent dose of asparaginase permits at least an additive therapeutic effect. The repeated use of this 2-day tandem schedule (MTX AsNase) permits the host to tolerate increasingly larger doses of MTX. These larger doses of MTX may have therapeutic benefit for the following reasons: 1) the steep dose-response relationship for MTX, 2) larger doses may overcome "transport-resistant" populations, and 3) larger doses may penetrate pharmacologic sanctuaries such as the blood-brain barrier. Trials of this combination in adults and children with advanced lymphoblastic leukemia, many of whom were previously treated with asparaginase and were refractory to conventional doses of MTX, resulted in complete remissions of 64% and 50%, respectively.

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Acute lymphoblastic leukemia (ALL) is the major clinical indication for the use of asparaginase either as a single agent or in combination chemotherapy (1). However, an appreciation of its schedule-dependent interactions with other antineoplastic agents, especially as regards its ability to protect normal organs from the toxic effects of methotrexate and cytosine arabinoside, may widen the spectrum of its clinical utility in the future.

A number of studies have shown that protein synthesis inhibitors or the deletion of an essential amino acid can attenuate the cytotoxic effects of antimetabolites and alkylating agents in tissue culture (2,5–7), normal (3) and tumor-bearing animals (4).

These observations should raise questions regarding the potential negative interaction between the protein synthesis inhibitor, asparaginase, and other drugs with which it might be combined for the treatment of acute leukemia. A negative therapeutic outcome in a standard cancer clinical trial is usually attributed to "drug resistance" unless the trial has been designed to include appropriately stratified and randomized populations intending to ask questions regarding schedule-dependency. In a recent report of a prospectively stratified and randomized clinical trial by CALGB, the delayed (sequential) administration of asparaginase after prednisone and vincristine produced superior remission durations compared to simultaneous treatment with the three drugs or pretreatment with asparaginase (median duration of remissions being 48+, 25, and 29 months, respectively) (8). Thus, for some combinations the same doses of the same drugs can have a profoundly different outcome depending on the schedule of drug administration.

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The effects of asparaginase and MTX in combination are schedule-dependent. When both drugs are administered simultaneously or if leukemic cells are pretreated with asparaginase, there is frank antagonism of the cytocidal effect of MTX by the asparaginase. Studies with the L5178Y/asn$^-$ murine lymphoblasts in cell culture clearly illustrate this pharmacologic antagonism (fig 1). In this experiment, cells were grown in suspension culture, treated with the drug(s) for the indicated period of time, then washed free of drug and cloned in soft agar for assessment of viability (10). A 3-hour exposure of L5178Y/asn$^-$ to MTX alone killed 90% of the cells. In contrast, the simultaneous exposure of the cells to MTX and a sublethal concentration of asparaginase (0.1 IU/ml) for 3 hours killed only 40% of the cells. Pretreatment of the cells with asparaginase alone for 3-6 hours completely attenuated the effect of a subsequent treatment with MTX.

Studies with the L5178Y ascites tumor in mice indicated that pharmacologic antagonism can occur in vivo if the mice are pretreated with asparaginase or if the two drugs are administered simultaneously (7,10-13). This antagonistic effect of asparaginase on MTX activity can be predicted by comparing the in vitro effect of MTX on ^{3}H-Udr incorporation into leukemic cell DNA before and at serial time intervals after a single parenteral dose of asparaginase (7). These biochemical studies suggested that the therapeutic effect of MTX for leukemic mice would be attenuated if the antifol was administered within 48 hours after asparaginase. However, beyond this time interval, the combination should result in at least an additive effect. These assumptions were confirmed with appropriate therapeutic experiments designed to investigate the "optimal" interval between the two drugs (7).

A summary of one series of experiments is shown in figure 2. A million L5178Y/asn$^-$ cells were injected intraperitoneally (ip) into male BDF$_1$ mice weighing 20-25 g. Ip injection of the drugs was begun 3 days after tumor implantation. The objectives of this experiment were to examine the effects of: 1) the sequence of drug administration, 2) the interval of time between the two drugs, 3) the interval of time between multiple courses of therapy, and 4) the dose-response effect of MTX, as they might affect the therapeutic and toxic outcome. The general protocol (fig 2) entailed the weekly administration of asparaginase, MTX or the combination which showed biochemical antagonism (7), ie, the A + M and A → M regimens, or the converse sequence, M → A. There was significant prolongation of mean survival time produced by the four weekly injections of either drug. The regimen in which biochemical antagonism is evident, ie, asparaginase followed by MTX 24 hours later, resulted in a subadditive improve-

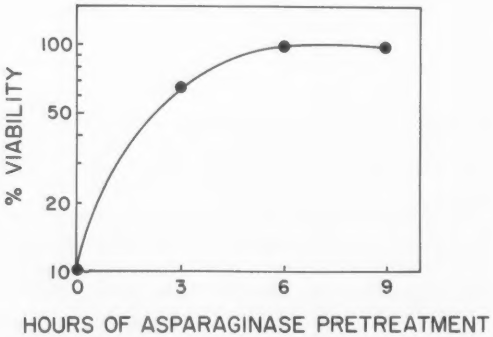

FIGURE 1.—Effect of pre or simultaneous treatment of L5178Y/asn$^-$ cells with asparaginase in cell culture on the cytotoxic effect of MTX. Each time point represents the effect of a 3-hour exposure to 5 × 10^{-7}M MTX in the presence of increasing exposure to asparaginase (0.1 IU/ml). (Reproduced with permission from Capizzi [7]).

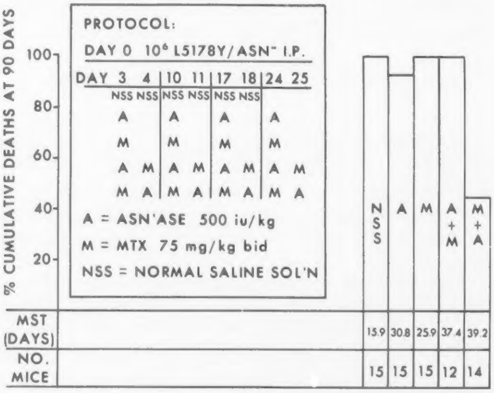

FIGURE 2.—Effect of sequence of drug administration on therapeutic efficacy. Leukemic mice were treated according to the protocol shown in the insert. MST = mean survival time; asn'ase = L-asparaginase. (Reproduced with permission from Capizzi [7]).

ment in mean survival time. Neither drug when used alone or in this sequence cured a significant number of mice, ie, resulted in 90-day survivors. In contrast, a simple reversal of the drug sequence (the M → A regimen) cured approximately 55% of the mice. The mean survival time of those mice which died reflected approximately additive therapeutic effects.

An important feature which contributed to the success of this program is the dose of MTX. The steep dose-response relationship for MTX against murine tumors has been well appreciated for a number of years (14). The prime limitation to using larger and possibly curative doses of MTX is toxicity to the normal organs of the host. In murine tumor systems a selective advantage to using higher doses of MTX with sparing of normal organs is clearly apparent with the use of sequential leucovorin (15), the enzyme carboxypeptidase G$_1$ (16) or the nucleoside, thymidine (17). Numerous clinical trials have utilized these "rescue" techniques and the general impression is that the higher doses of MTX are beneficial in selected situations (18–20). The theoretical benefits in using high dose MTX are at least threefold: 1) a consideration of the dose-response relationship (14), 2) the possibility of overcoming therapeutic refractoriness as a result of impaired cellular transport of the drug (21), and 3) pharmacokinetic reasons, ie, higher blood levels allow the penetration of the blood-brain (22,23) and possibly other blood-organ barriers. When given 24 hours before or after larger doses of MTX, asparaginase will attenuate some of the toxic effects of MTX, allowing the host to tolerate larger doses of the antifolate. Thus, the improved therapeutic effects of the combination are not only the result of the individual effects of the drugs but also the effects of schedule-dependent drug-drug interactions.

Reasons for the asparaginase-induced attenuation of MTX effect have been explored in the laboratory. Carrier-mediated transport is a well-defined determinant of MTX effect in murine tumor cells (21). MTX transport into L5178Y/asn$^-$ cells was determined in vitro at serial time intervals after a single parenteral dose of asparaginase (fig 3). In lymphoblasts which are sensitive to asparaginase, the enzyme will attenuate the cellular uptake of MTX. This inhibitory effect on MTX uptake is maximal 24 hours after asparaginase and returns to control values 96 hours later. Whether this impairment is a growth or cell cycle-dependent effect or a direct effect of asparaginase on the cell membrane remains to be established. Amino acid depletion may cause the cells to enter a stationary phase of growth (7,24), a phase wherein MTX transport and its ultimate cytocidal effect is attenuated (25).

In sensitive tumor cells asparaginase causes a profound inhibition of DNA synthesis. As with all antimetabolites, MTX is most toxic against those tumors composed of cells which are actively synthesizing DNA (25,26). Thus, the asparaginase-induced inhibition of DNA synthesis may significantly attenuate MTX activity.

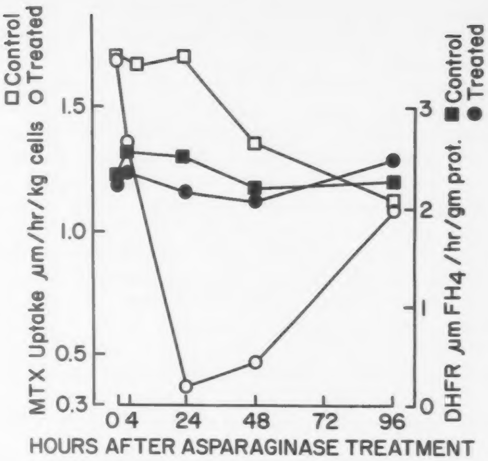

FIGURE 3.—Effect of asparaginase pretreatment of mice on MTX uptake into L5178Y/asn$^-$ cells and cellular specific activity of dihydrofolate reductase (DHFR). Leukemic mice were given a single ip injection of 250 IU asparaginase/kg. Cells were harvested at serial time intervals thereafter and exposed to ^{3}HMTX in vitro; values shown reflect steady-state concentrations of MTX. Control = ^{3}HMTX uptake and DHFR specific activity in cells taken from saline-treated mice. (Reproduced with permission from Nahas and Capizzi [34]).

The cytostatic effect of amino acid deficiency and protein synthesis inhibition is mediated by a block at the G$_1$S border (24). Consequently, the timed administration of MTX intended to coincide with the release of this asparaginase-induced block in cell cycle traverse may capture the cells as they enter S phase, thus enhancing the overall cytotoxic effect of MTX. These interactions may contribute to the observed schedule-dependency between asparaginase and MTX.

The above preclinical observations raised certain questions regarding the clinical use of this combination. This was of special concern since most of the patients with ALL who would be candidates for a "second line" protocol would have already been treated with MTX during a previous remission. In routine clinical practice a patient who has sustained a relapse while being treated with a given drug is usually not given a new combination which includes the same drug. Certain circumstances, however, may mitigate this rule. If a true dose-response relationship exists for the drug and a given disease and provided that the "ceiling dose" of the drug had not already been given, the drug may then be reintroduced at a higher dose, if possible. In the case of MTX, host toxicity limits the achievement of this antitumor "ceiling dose." Since the preclinical studies in mice indicated that the dose of MTX could safely be escalated when given in conjunction with asparaginase, a dose escalation scheme was introduced into the clinical protocol. In the preclinical studies it was shown that simultaneous or antecedent asparaginase attenuated MTX effect. Thus, while the converse sequence, MTX followed by asparaginase, would be useful for the first course of the combination, the question arose regarding the optimal time interval between multiple courses of the two drugs. The asparaginase of course 1 (M → A) would essentially be pre-MTX treatment for course 2. A short interval between courses of therapy would effectively "sandwich" a dose of MTX between two doses of asparaginase which could effectively attenuate MTX activity. To determine this time interval, human leukemic lymphoblasts were exposed to pulse doses of MTX in vitro and the inhibitory effect of MTX on ^{3}H-UdR incorporation into leukemic cell DNA was determined before and in a serial fashion after a single dose of asparaginase (7,27). While this test is not a "MTX-sensitivity" test by any means, it does reflect certain important biochemical parameters which have been correlated with MTX cytotoxicity, viz, cellular uptake of the drug and inhibition of the enzyme, dihydrofolate reductase. Using this biochemical observation as an index of MTX effect on the cell, the "optimal" interval between a dose of asparaginase and the subsequent dose of MTX for human lymphoblasts was determined to be no less than 9–10 days (27,28). These data would suggest, then, that weekly courses of MTX and asparaginase might be less effective than a 10–14-day schedule. Thus, the protocol outlined in table 1 was developed.

TABLE 1.—MTX/asparaginase protocol for refractory ALL

I. Initial		
Day 0	A'ase*	6000 IU/m^2 im
II. Continuous		
Day 10	MTX	60 mg/m^2 iv
Day 11	A'ase	6000 IU/m^2 im
Repeat MTX-A'ase q 9–10 days Mon-Tues alternating with Thurs-Fri		
III. Escalate MTX		
Increase dose of MTX by 25%–50% with each course to point of minimal toxicity; then maintain with a 25% reduction		

*A'ase = asparaginase

A summary of five clinical trials which have reported the use of this combination for the treatment of children and adults with refractory ALL is shown in table 2. Almost half of the 36 adults had been previously treated with MTX; none had prior exposure to asparaginase. Twenty-three of the 36 (64%) patients sustained a complete remission (23,29). Of the initial ten adults reported by Capizzi et al (23), nine had relapsed on MTX-containing combinations. The one patient who had not had previous treatment with MTX was a woman who failed to enter complete remission with prednisone and vincristine. A remission induction was achieved with MTX and asparaginase and she was then maintained on high dose intermittent MTX plus asparaginase; she continues in her first remission for 8+ years. Of 39 children who had extensive prior exposure to both MTX and asparaginase, approximately 50% have entered complete remission (27,30,31). Several of these reports used a 7-day interval between courses in comparison to the 10-day interval that was suggested by the biochemical studies. The shorter treatment interval may have several disadvantages: 1) the MTX may be given at a time when the previous dose of asparaginase may block MTX effect on the tumor cell, and 2) host toxicity may actually be enhanced by the shorter interval since recovery of normal organs from asparaginase effect may be at its peak at this time. This might explain the lower maximally tolerated dose of MTX reported for those patients treated with the 7-day interval compared to the 10–14-day interval between courses. This lower dose of MTX may, in turn, affect remission duration if tumor cell kill follows a dose-response relationship. In general, the combination has been most effective in patients who have not had prior exposure to asparaginase. This observation may be explained in part by the previously observed rapid development of tumor cell resistance to asparaginase (1).

In the initial clinical trials of this combination for adults and children, the dose of MTX was deliberately escalated to the point of maximal tolerance since 37 of the 40 patients had relapsed while being treated with lower doses of MTX (30,34). Figure 4 relates the dose of MTX to the hematocrit and absolute granulocyte, lymphocyte, and platelet counts. Despite the rapid escalation from an average starting dose of 60 mg/M^2 to an average plateau dose of 350 mg/M^2, the absolute granulocyte and platelet count remained in excess of 2000/mm^3 and 200,000/mm^3, respectively.

The ability of patients to tolerate larger doses of MTX coupled with the observed remission rate in patients with advanced disease suggests that asparaginase may improve the therapeutic index of MTX (32). This effect is mediated through a schedule-dependent interaction which allows both drugs to exert their antileukemic effect along with an asparaginase-induced

TABLE 2.—MTX/asparaginase treatment of refractory ALL

Series	No.	Cr	Previous MTX	Previous A'ase*	Average maximal dose of MTX (mg/m²)	Refs.
I. Adults						
Capizzi et al	10	8	9	0	350	30
Yap et al	26	15	7	0	200	36
II. Children						
Lobel et al	12	4	11	8	361	34
	40	Maint.†	37	5		
Amadori et al‡	17	10	17	17	NS§	37
Harris et al	10	6	10	9	230	38
	4	Maint.	4	4		

*A'ase = asparaginase.

†Maint. = MTX/asparaginase used as maintenance therapy after inductions with other regimens.

‡Median age for group 11 years (range 4–31).

§NS = not stated.

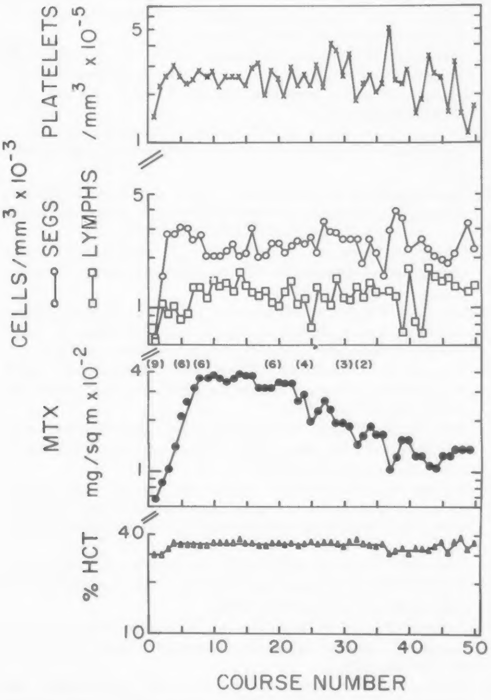

FIGURE 4.—Hematologic tolerance to MTX/asparaginase in patients with ALL through 50 courses of therapy separated by 9-10 day intervals. Patients were treated according to the protocol shown in table 1 except that the dose of asparaginase was 500 IU/kg for each course. ( ) = number of patients at each time point.

attenuation of MTX toxicity against normal bone marrow and gastrointestinal tract, ie, schedule-dependent selective cytotoxicity.

A plot of maximally tolerated dose of MTX versus duration of remission suggests a dose-response relationship (fig 5).

The larger intermittent doses of MTX made possible with this combination may have further clinical utility for pharmacokinetic reasons. The larger systemic doses of MTX given three times a month penetrate the blood-brain barrier (table 3) and thus may be beneficial in preventing CNS leukemia. In addition, systemically administered asparaginase has been shown to have a therapeutic effect on CNS leukemia (33).

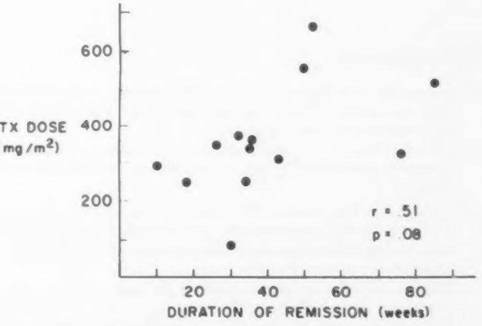

FIGURE 5.—Duration of complete remission with MTX/asparaginase maintenance therapy related to maximally tolerated dose of MTX in 13 patients. Mean dose attained was 361 mg/m². (Reproduced with permission from Lobel et al [27]).

Table 3.—Effect of dosage level of MTX on the concentration of this agent in serum and CSF

Patient	MTX dose		Level of MTX(M)		Time (hr)*
	Mg/m^2	Total (mg)	Serum	CSF	
E.C.	300	540	1.06×10^{-7}	1.4×10^{-7}	24
V.E.	600	900	2.75×10^{-7}	1.18×10^{-7}	24
W.K.	450	900	1.05×10^{-6}	3.0×10^{-7}	19
W.K.	600	1200	9.5×10^{-7}	5.0×10^{-7}	22

*Hours after the intravenous injection of MTX.

In the report by Lobel et al (27), five of 18 children developed CNS leukemia during remissions prior to the use of MTX/asparaginase; all had had standard "CNS prophylaxis" during their first remission. The patients achieved CNS remission with standard therapy but were not given maintenance intrathecal therapy during treatment with MTX and asparaginase. One of the five patients had a recurrent CNS relapse and one other patient developed subsequent CNS leukemia during treatment with MTX and asparaginase.

In summary, asparaginase may have a unique role in combination chemotherapy. As with other protein synthesis inhibitors it may attenuate the cytotoxic effects of antimetabolites on normal organs as well as tumor cells. However, with the appropriate schedule the cytotoxic effect of the two drugs against the leukemia cells will be preserved and the patient may tolerate larger doses of MTX. These larger doses of MTX may, in turn, enhance the therapeutic effects against the leukemia and allow the drug to penetrate pharmacologic sanctuaries. A further understanding of the mechanism involved in this drug-drug interaction may further enhance this selective cytotoxicity.

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Asparaginase and Amino Acids in Cancer Therapeutics[1]

James F. Holland,[2,3,*] and Takao Ohnuma,[2,3] and Cancer and Leukemia Group B Investigators

SUMMARY

Asparaginase was found ineffective in improving induction frequency of acute lymphocytic leukemia of children from vincristine and prednisone, but prolonged remission duration. Prolongation of remission duration was accomplished more effectively and at lower toxic cost by administration of asparaginase after the vincristine and prednisone treatment had been completed. Asparaginase is markedly more effective against human leukemic T-cells in vitro than against several types of B-lymphocytes. This is consistent with in vivo experience. Leukemic T-cells resistant to asparaginase have been induced in vitro. Deficiency of other amino acids causes absolute cessation of RNA biosynthesis, constituting evidence for other amino acid targets critical to leukemic cell survival.

[Cancer Treat Rep 65 (Suppl 4):123-130, 1981]

Asparaginase is a unique anticancer drug. It is unique in being a foreign protein, and in its induction of a specific biochemical depletion syndrome, asparagine deficiency, which mediates its effect (fig 1). The critical observation of Kidd (1) that guinea pig serum (used for its complement content) led to antileukemic activity in mice prompted a search for the mechanism of this effect. Broome's (2,3) identification of the serum constituent as asparaginase led to a major thrust of activity since this was a specific nutritional link to leukemic cell growth. Early studies with asparaginases from yeast, guinea pigs, other rodents, and chicken liver were superseded by the isolation of asparaginase from *Escherichia coli* (4) and later from *Erwinia carotovora* (5). The spectrum of useful asparaginase antineoplastic activity has narrowed to acute lymphocytic leukemia in man (6,7), although lymphomas in dogs are sensitive (8). A single case of acute myelocytic leukemia has been observed in which one large dose of asparaginase evoked complete remission (9).

Asparaginase has a spectrum of toxicity dependent on its ability to cause immediate cessation of protein biosynthesis in tissues dependent on exogenous asparagine, since virtually all proteins contain asparagine. Thus inhibition of hepatic biosynthesis of albumin, of hepatic enzymes, of pancreatic secretion of insulin and perhaps of trypsin inhibitors, leads to hypoalbuminemia, hepatotoxicity, hyperglycemia (even to the point of hyperosmolar coma), and acute pancreatitis (6,7). Coagulopathy reflects interference with the metabolism of proteins involved in coagulation. Induced asparagine deficiency alters cerebral and emotional functions, and deficiencies in psychomotor performance have also been described (10). Nausea and vomiting are commonplace. Chills may relate to the foreign protein per se or conceivably to minute quantities of bacterial pyrogen. The foreign protein can evoke immunologic response of minor degree or can provoke anaphylaxis. Asparaginase should not be administered by someone who has not contemplated in advance what steps should be taken if anaphylaxis appears (table 1).

The protein nature of asparaginase alters its pharmacokinetics markedly from those of common crystalloidal drugs. The plasma disappearance half-time is prolonged. Higher doses produce longer detectable blood levels because the half disappearance time is independent of dose (fig 2). When multiple doses are given, the

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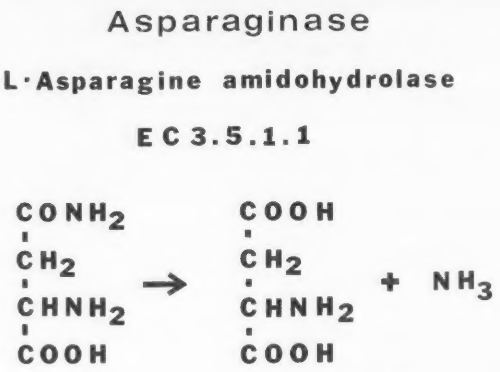

$$CONH_2-CH_2-CHNH_2-COOH \rightarrow COOH-CH_2-CHNH_2-COOH + NH_3$$

FIGURE 1.—The biochemical effect of asparaginase.

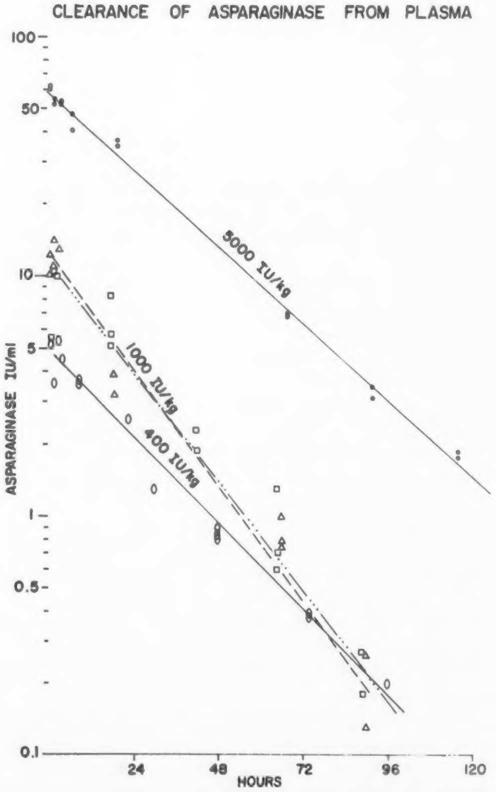

FIGURE 2.—Serum levels of single doses of *E. coli* asparaginase. Studied in replica in four patients.

TABLE 1.—Causes of induction deaths (CALGB protocol 7111)

Causes of death	No asp* (84)† %	Prior (103)† %	Simult. (115)† %	Subseq. (344)† %
Sepsis	2.4	5	2.6	1.5
Hemorrhage	1.2			1.2
Anaphylaxis		3	2.6	
Necrotizing enteropathy			0.9	0.3
Nonketotic hyperglycemia			2.6	
Uric acid nephropathy				0.6
Meningeal leukemia				0.3
Unknown-sudden			0.9	0.3

*asp = asparaginase.
†Patients entered.

drug may accumulate with appreciable levels present for longer than 1 week (11) (fig 3).

The asparaginase from *Erwinia carotovora* has essentially identical activity with that from *E. coli,* although the proteins are immunologically distinct. *Erwinia carotovora* enzyme has been administered successfully after anaphylaxis to the *E. coli* enzyme without evidence of cross-reactivity (12). At similar dose levels, the plasma clearance is nearly identical (fig 4).

The Cancer and Leukemia Group B (CALGB) has investigated asparaginase in a large number of protocols. In 1969 the enzyme was studied in patients with acute lymphocytic leukemia in relapse from other chemotherapy (13). Asparaginase was given by weekly injec-

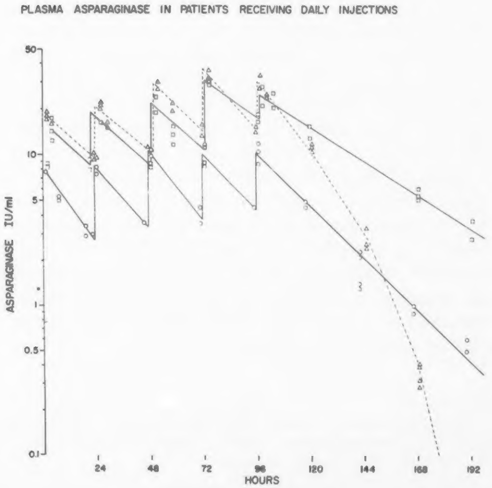

FIGURE 3.—Serum levels of *E. coli* asparaginase after multiple doses. Studied in replica in three patients.

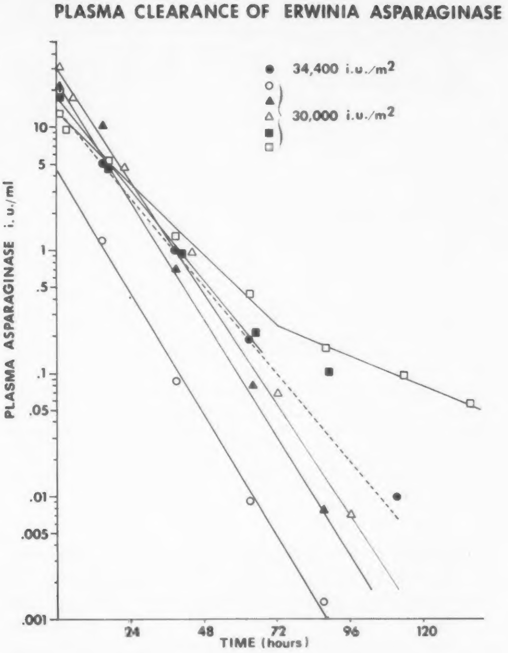

FIGURE 4.—Serum levels of asparaginase from *Erwinia carotovora.* Given in single doses to six patients.

tion or by daily administration, alone or in combination with vincristine, prednisone, and daunorubicin. The weekly dose was 5000 IU/kg and the daily dose 1000 IU/kg. No important difference in response frequency occurred between the two schedules of administration, although the four-drug combination was twice as active as asparaginase alone (fig 5).

Based on the activity of asparaginase in the relapsed disease, we undertook a study of asparaginase as a preinduction chemotherapeutic agent in children with untreated leukemia (M. Weil and O. Glidewell, Unpublished CALGB data). When the endpoint was judged as attainment of M1 marrow status, 5 days of asparaginase, 1000 IU/kg/day did not influence the remission induction frequency from vincristine and prednisone, or from vincristine, prednisone, and daunorubicin (fig 6).

The role of induction treatment in subsequent response duration in patients with acute lymphocytic leukemia has been one important strategy in stepwise improvement of therapy. In the same study program wherein 5 days of asparaginase made no difference in remission frequency, no impact on remission duration could be appreciated in children at standard risk (fig 7) or in those with acute leukemia at increased risk (less than age 2, greater than age 8, or leukocyte count greater than 30,000/μl). The paradox of a demonstrably active inducing agent neither increasing the frequency nor the duration of response in a sensitive neoplasm led us to increase the intensity of asparaginase administration.

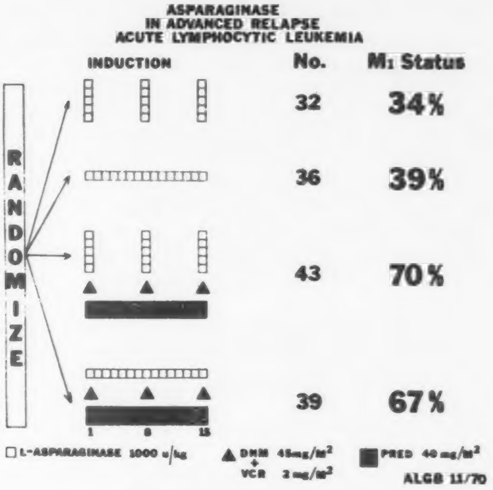
ASPARAGINASE
IN ADVANCED RELAPSE
ACUTE LYMPHOCYTIC LEUKEMIA
INDUCTION
No.
M1 Status
RANDOMIZE
32
34%
36
39%
43
70%
39
67%
1
8
15
L-ASPARAGINASE 1000 u/kg
DNM 45mg/M²
+
VCR 2 mg/M²
PRED 40 mg/M²
ALGB 11/70

FIGURE 5.—Induction frequency from asparaginase, given alone or in combination by two different schedules, in patients with acute lymphocytic leukemia in relapse under 20 years. CALGB protocol 6911.

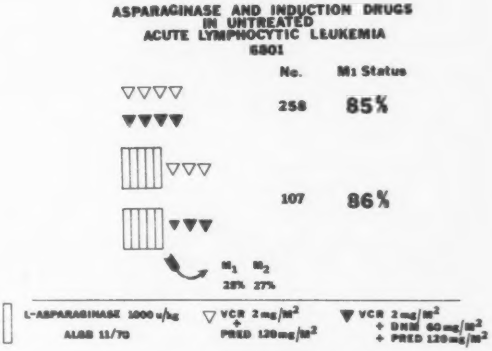
ASPARAGINASE AND INDUCTION DRUGS
IN UNTREATED
ACUTE LYMPHOCYTIC LEUKEMIA
6801
No.
M1 Status
258
85%
107
86%
M1 M2
25% 27%
L-ASPARAGINASE 1000 u/kg
ALGB 11/70
VCR 2 mg/M²
+
PRED 120mg/M²
VCR 2 mg/M²
+ DNM 60 mg/M²
+ PRED 120mg/M²

FIGURE 6.—Failure of 5 days of asparaginase to increase remission frequency of acute lymphocytic leukemia. CALGB protocol 6801. The remission frequency for the two chemotherapeutic inductions combined is the same as when they are preceded by asparaginase. The 5-day asparaginase regimens alone led to 52% complete or partial remission marrow status (indicated by arrow), which we converted to 86% complete remission marrow status by the vincristine and prednisone, with or without daunorubicin.

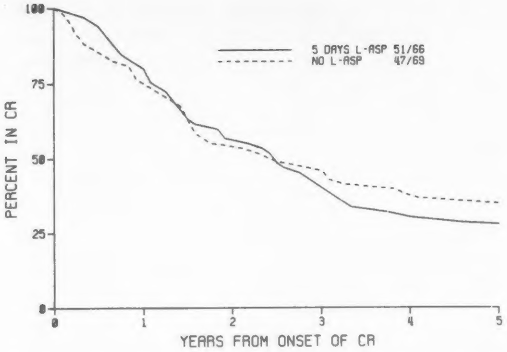

FIGURE 7.—Complete remission durations with and without asparaginase in standard risk acute lymphocytic leukemia. CALGB protocol 6801.

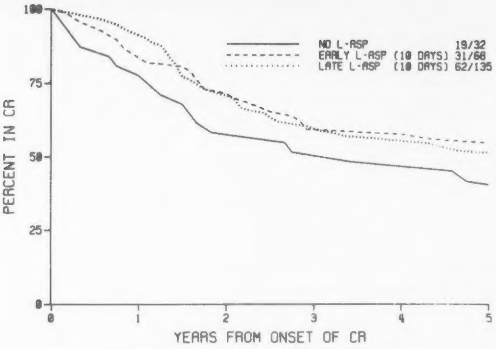

FIGURE 9.—Effect of schedule of asparaginase administration on complete remission duration in standard risk children with acute lymphocytic leukemia treated with chemotherapy plus asparaginase early (before or simultaneously) or later (after vincristine and corticosteroid). p = N.S., not statistically significant. CALGB protocol 7111.

In protocol 7111 asparaginase dose was increased to 1000 IU/kg/day for 10 consecutive days (fig 8). Because of alterations which this evoked in protein synthesis, asparaginase was given before, during, or after vincristine and prednisone induction (14). This was done on the proposition that asparaginase might affect cells differently which were cycling, uninfluenced by prior drug therapy, undergoing simultaneous attack, or which had been exposed to vincristine and prednisone. A simultaneous control of vincristine and prednisone alone without asparaginase was also studied. Although additional random choices of maintenance regimens were a part of the study design, these have been lumped for consideration of the asparaginase effect. In children with standard risk acute lymphocytic leukemia, asparaginase prolonged remission duration by approximately 1 year (fig 9). In the fifth year where the relapse curves are becoming more nearly in plateau, indicative of cure expectancy, there are approximately 10% more asparaginase-treated patients than patients who did not receive the enzyme. There is no distinction among those who received asparaginase after vincristine-prednisone (late) and those who received it antecedent to or simultaneously with the vincristine and prednisone (early). In sharp contrast, patients with increased risk acute lymphocytic leukemia showed benefit from asparaginase only when it was administered after vincristine and prednisone (fig 10). Those who received the drug before or during the other induction treatments have a relapse pattern identical to those without asparaginase administration. The mechanism for this phenomenon is not immediately apparent save

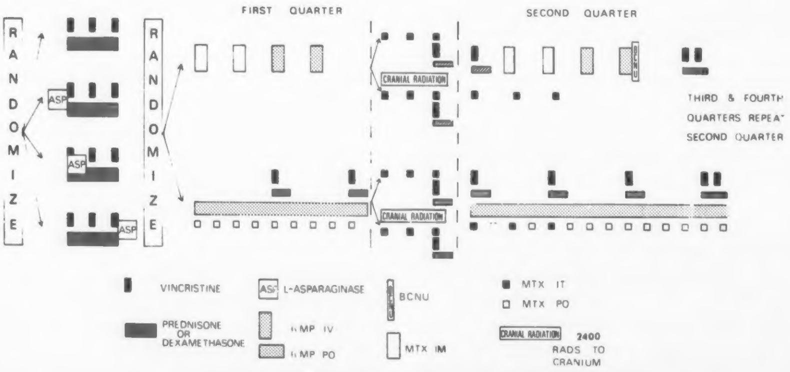
RANDOMIZE
ASP
ASP
ASP
RANDOMIZE
FIRST QUARTER
CRANIAL RADIATION
CRANIAL RADIATION
SECOND QUARTER
BCNU
THIRD & FOURTH QUARTERS REPEAT SECOND QUARTER
VINCRISTINE
PREDNISONE OR DEXAMETHASONE
ASP L-ASPARAGINASE
6MP IV
6MP PO
BCNU
MTX IM
MTX IT
MTX PO
CRANIAL RADIATION 2400 RADS TO CRANIUM

FIGURE 8.—Schema of CALGB protocol 7111.

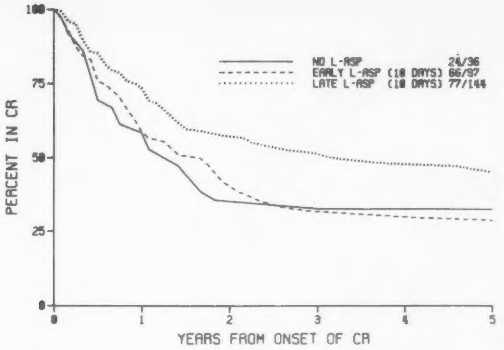

FIGURE 10.—Effect of schedule of asparaginase (asp.) administration on complete remission duration in increased risk children with acute lymphocytic leukemia. Late asp. v. Early asp. p = <.001, Early asp. v. no asp. p = N.S. CALGB protocol 7111.

for the obvious altered metabolism of vincristine and prednisone which might derive from hepatic enzymatic changes resulting from the induced asparagine deficiency.

In addition to the compromised effect of early asparaginase in patients at high risk, the administration of the enzyme before or with vincristine and prednisone led to significantly higher mortality (8% and 9.6%) during induction than did the vincristine and corticosteroid alone (3.6%) or the late asparaginase (4.2%). Anaphylaxis, uncontrolled diabetes and possibly sepsis account for this difference in mortality (table 1). Based on these data, the CALGB has adopted the intravenous use of asparaginase *after* vincristine and corticosteroid or vincristine, corticosteroid, and daunorubicin induction regimens in acute lymphocytic leukemia.

A major understanding of asparaginase activity has derived from the in vitro studies of Ohnuma and coworkers (15). In the presence of asparagine in an otherwise complete medium, two T-cell lines (MOLT-3 and RPMI-8402), five B-cell lines (RPMI-1788, RPMI-8422, B411-4, B46M, and DND-39A), and three non-T/non-B cell lines (NALL-1, NALM-16, and K562) grow logarithmically. When asparagine is eliminated from the medium the two T-cell lines are unable to sustain viability, whereas the B-cell and non-T/non-B cell lines are unaffected (fig 11). From these observations it was concluded that the two T-cell lines (leukemic) had an obligatory requirement for asparagine, whereas the B-cells and non-T/non-B cells contained asparagine synthetase which could satisfy their needs. These same conclusions explain the thousandfold difference in susceptibility to asparaginase of the two T-cell lines (fig 12). By feeding on a medium with progressively lowered concentrations of asparagine the T-cells can adapt over many generations to grow in lesser quantities and then in the absence of the amino acid, thereby becoming resistant to asparaginase (MOLT-3/Asn) (16) (fig 13). These cells were shown to have greatly increased asparagine synthetase activity approaching that of B-cells (fig 14). These observations with asparaginase represent the first instance of induced biochemical resistance of human leukemic cells in vitro. The system offers a challenge for further understanding of the biochemical pathogenesis of and therapeutic approaches to resistance, one of the major problems of chemotherapy.

One approach to new nutritional and chemotherapeutic targets in leukemic cells is to follow the lessons of specific amino acid requirements exemplified by asparagine. Indeed, several amino acids appear to be more critical for cell viability than is asparagine (17). In one critical parameter of cell viability, the synthesis of RNA in freshly isolated leukemic cells, baseline activ-

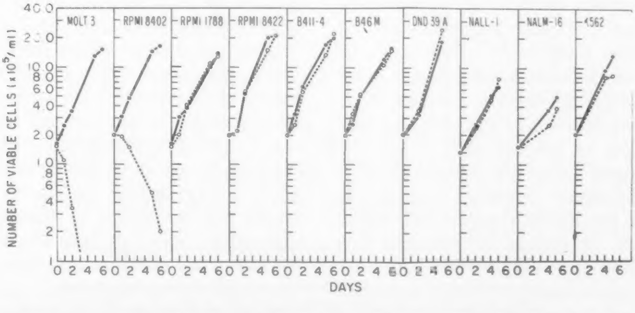

FIGURE 11.—Growth of T-cells (two left panels), B-cells and non-T/non-B cells in vitro with (solid line) and without (broken line) asparaginase. From Ohnuma et al (15).

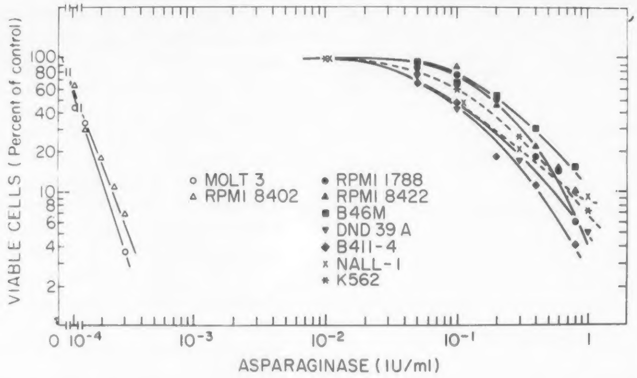

FIGURE 12.—Effect of asparaginase on T-cell, B-cell and non-T/non-B cell growth in vitro. Culture flasks contained an initial viable cell concentration of 2 × 10^5 cells/ml of RPMI-1640 medium with 10% fetal calf serum plus different concentrations of the enzyme. After 5 days' growth, the viable cells were counted and plotted. We assumed the viable cell concentration in the enzyme-free control flask to be a unity. From Ohnuma et al (15).

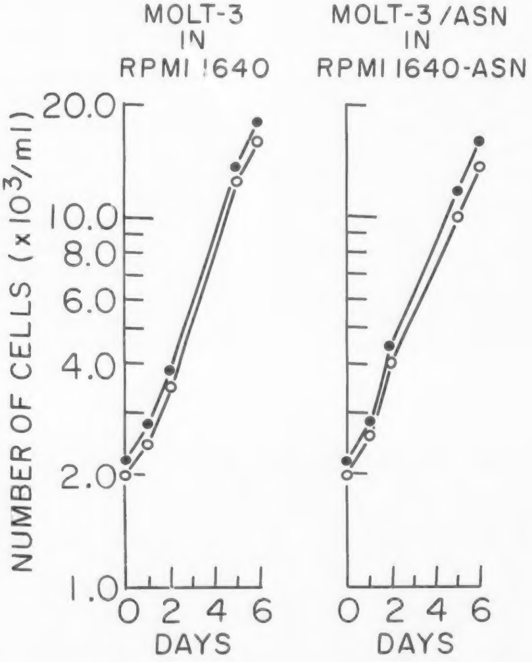

FIGURE 13.—Total (filled circle) and viable (open circle) cell growth of parent MOLT-3 cells in RPMI-1640 medium plus 10% fetal calf serum (left panel) and of MOLT-3/Asn in RPMI-1640 medium devoid of asparagine plus 10% dialyzed fetal calf serum. Note that MOLT-3/Asn cells grow as avidly as the parent cells in the absence of asparagine in the medium. From Ohnuma et al (16).

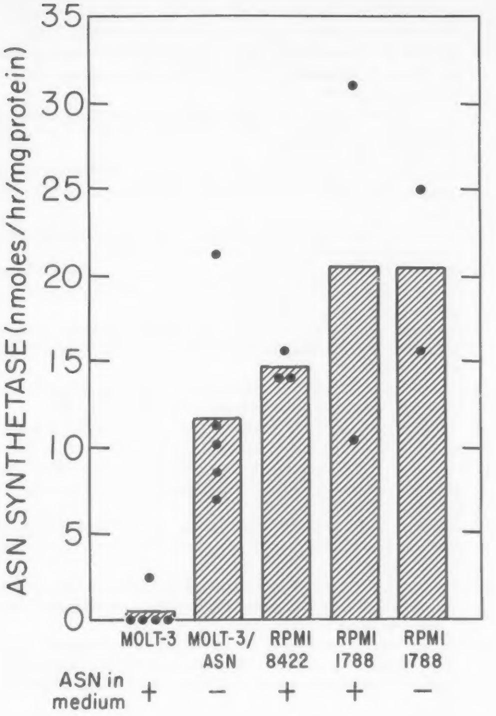

Figure 14.—Asparagine synthetase activity of MOLT-3, MOLT-3/Asn, RPMI-8422 and RPMI-1788. The "+" and "−" indicate presence and absence of asparagine in the RPMI-1640 medium plus 10% dialyzed fetal calf serum. The results of each determination are shown as filled circles; the bars represent the average. From Ohnuma et al (16).

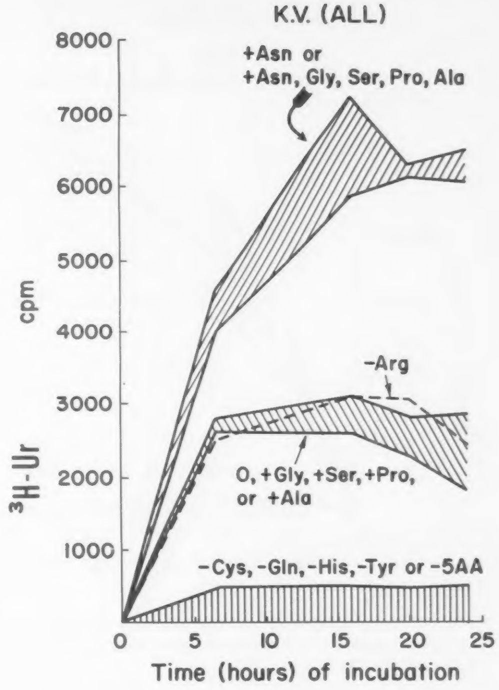

Figure 15.—Tritiated uridine uptake into RNA of fresh human acute lymphocytic leukemic cells as affected by amino acid deficiencies in the medium. Leukemic cells, 10^6/ml, were incubated at 37°C for 24 hours in 2 ml of medium containing Eagle's minimal essential medium with Hank's balanced salt solution, with additions or subtractions of single amino acids or mixture and ^{3}H-uridine 1μCi/ml. From Ohnuma et al (17).

ity over 24 hours of incubation (shown in the middle hatched area of fig 15) is unaffected in basal medium by the addition of glycine, serine, proline, or alanine. The addition of asparagine or of asparagine plus these four amino acids augments RNA biosynthesis to significantly higher levels. The removal of arginine from the medium returns the synthesis levels back to baseline, but the removal of either cysteine, glutamine, histidine, or tyrosine or all five of these amino acids reduces RNA biosynthesis to negligible quantities. Thus, one might conclude that cysteine, glutamine, histidine, or tyrosine depletion might cause more significant injury than asparagine depletion, an approach which begs for exploitation by a search for specific purified amino acid catabolizing enzymes, or for unique antimetabolites to these amino acids. The validity of this interpretation from in vitro findings is supported by an identical experiment performed in freshly isolated acute myelocytic leukemia cells. The addition of asparagine or of asparagine plus glycine, serine, proline, and alanine did not materially increase RNA biosynthesis. The removal of arginine inflicted minor damage to the cells which asparagine deficiency (the zero control medium compared to the asparagine-supplemented medium) did not. Again, however, depletion of cysteine, glutamine, histidine, tyrosine or a combination of these amino acids from the medium abolished RNA biosynthesis (fig 16).

The strategy of treatment of cancer cells does not demand that drugs be nontoxic for normal cells. Modifi-

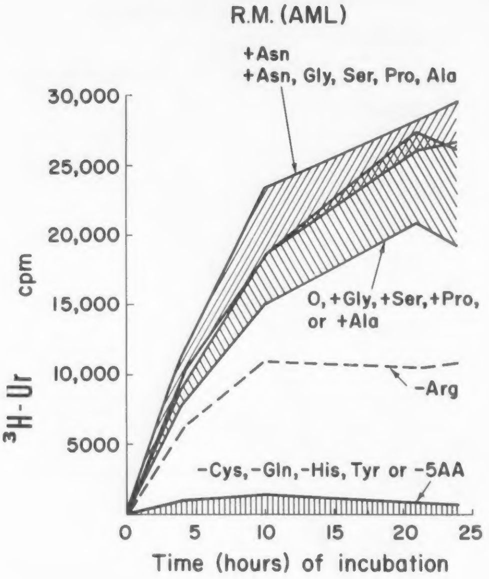

Figure 16.—Tritiated uridine uptake into RNA of fresh human acute myelocytic leukemic cells as affected by amino acid deficiency in the medium. From Ohnuma et al (17).

cation of essential nutrient levels may injure tumor cells more rapidly or more profoundly than normal cells. Even in the presence of equal injury, more efficient recovery by normal tissues may constitute the essence of the therapeutic margin.

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